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Establishing a gene expression system to screen the effects of dietary fibers and their metabolites on selected aspects of colon cancer development

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Contents

I Acknowledgments

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II Abbreviations

18S rRNA	18S ribosomal RNA
ACTB	Beta actin
AMV	Avian myeloblastosis virus
ANOVA	Analysis of variance
APC	Adenomatous polypois coli
ATCC	American Type Culture Collection
B2M	Beta-2-microglobulin
C_T	Threshold cycle
cDNA	Complementary DNA
DMEM	Dulbecco modified Eagles minimum essential medium
FBS	Fetal bovine serum
FRET	Fluorescence resonance energy transfer
GAPDH	Glyceraldedyde 3-phosphate dehydrogenase
MEM	Eagles minimum essential medium
MGB	Minor groove binding molecule
MMLV	Moloney murine leukemia virus
mRNA	Messenger RNA
NEAA	Non-essential amino acids
NFE2L2	Nuclear factor erythroid 2-related factor 2
NFG	Non-fluorescent dye
NF-κB	Nuclear factor-κB
NF-κB1	Nuclear factor-κB p105
NRT	Non-reverse transcriptase sample
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PTGS2	Prostaglandin H2 synthase 2
qPCR	Real-time PCR
RPMI	Roswell Park Memorial Institute medium
RT-PCR	Reverse transcription PCR
RT-qPCR	Quantitative real-time reverse transcription PCR
SCFA	Short chain fatty acids
Tukey HSD	Tukey honest significant difference

III Preface

This master's thesis was performed at Nofima – Norwegian Institute for Food, Fisheries, and Aquaculture Research – between February 2014 and June 2015 as part of the research project Optifiber (KPN-NFR 224819/E40).

This thesis is structured as follows. The introduction describes, along general lines, colon cancer and dietary fibers as the background of the study. Also the theory of real-time quantitative RT-PCR (RT-qPCR) is described there. The introduction is meant to support readers unfamiliar with RT-qPCR in understanding the following parts of the thesis. The adapted RT-qPCR method, which is necessary for establishing a test system for analyzing the mechanisms behind the potential protective effects of the dietary fibers, is described in the methods. The adaption of the RT-qPCR method was the main aim of the thesis. The application of the test system to one specific dietary fiber is detailed in the results. The strengths and weaknesses of the test system are then discussed together with the results of the application. The thesis ends with a conclusion and suggestions for future studies.

This thesis was typeset using LaTeX including the ChemFig package for chemical structures.

IV Summary

A higher intake of dietary fibers is associated with a decreased risk of colorectal cancer. The molecular mechanisms of the protective effect of dietary fibers against colorectal cancer are, however, still largely unknown.

In this thesis, a simple screening test system was established for studying the molecular mechanisms behind the potential protective effects of the dietary fiber type β-glucan which is the main component of the product dispersible wellmune and the fiber metabolite butyrate. In the test system the, changes in gene expression of *NFκB1*, *PTGS2*, and *NFE2L2* induced by dispersible wellmune or butyrate treatment in the human colon carcinoma cell lines HT-29 and HCT-116 were investigated. For establishing the test system, a quantitative real-time reverse transcription PCR (RT-qPCR) method was adapted which included the development of an optimized RNA isolation, cDNA synthesis, PCR method, and reference gene selection for data normalization. The stability of the four reference genes *GAPDH*, *18S rRNA*, *B2M*, and *ACTB* after treatment with dispersible wellmune or butyrate was analyzed. Data analysis using ANOVA combined with a Tukey HSD test and the stability validation programs BestKeeper and NormFinder indicated that *18S rRNA*, *B2M*, and *ACTB* were the most stable reference genes while *GAPDH* was stated as unstable and not suitable for normalization. Further evaluations confirmed that *GAPDH* was indeed not suitable for data normalization in HT-29 cells. However, this was not the case in HCT-116 cells.

By using the adapted RT-qPCR method it was shown that a treatment with dispersible wellmune or butyrate led to a down-regulation in gene expression of *NFκB1* and *PTGS2* and an up-regulation of *NFE2L2* in HT-29 cells after 24 hours treatment time. The best treatment time was 24 hours, all gene expression alterations were observable after this time period which was not the case after three and six hours treatment time. The only exception was the down regulation of *NFE2L2* after six hours with butyrate treatment, which was no longer visible after 24 hours of treatment. The gene expression alterations observed in HCT-116 cells after 6 hours of incubation with dispersible wellmune or butyrate followed the same trend as observed for HT-29 cells. The HCT-116 cell line does not express the *PTGS2* gene.

For establishing the screening test system and utilizing the RT-qPCR method, the experimental setup with the above mentioned steps needed to be rigorously controlled. The observed alterations in the gene expression of the target genes when using the RT-qPCR method may be one potential mechanism of how dietary fibers protect against the development of colon cancer.

1 Introduction

1.1 Background: Colon cancer and dietary fiber

1.1.1 Brief overview

In 2014, colorectal cancer was the fourth most common type of cancer in the world (Stewart and Wild, 2014). According to Slattery et al. (1998), the Western-style diet characterized by a high proportion of red or processed meat and refined grains, is associated with an increased risk of colorectal cancer, while a increased consumption of whole grains and vegetables will reduce the risk of colorectal cancer. Already Burkitt (1969) found that the intake of dietary fibers is inversely associated with the risk of developing colorectal cancer. Becker et al. (2004) have evaluated the link between dietary fiber intake and decreased risk of colon cancer development as moderately likely. Even though many studies have shown a protective effect of dietary fiber, there are still controversy results. These differences may be due to the use of different fiber sources. Dietary fibers are a heterogeneous group of carbohydrate polymers with different structures. Consequently, different dietary fibers may have different protective effects. However, the molecular mechanisms behind dietary fibers potential protective effect on colon cancer development are still poorly understood. The aim of this thesis was therefore to create a test system that can be used to screen different dietary fibers for their effects and at the same time increase the understanding of the molecular mechanism that form the basis of the protective effect of dietary fibers in colon cancer development.

1.1.2 Function of the gut

The human gut, also known as gastrointestinal tract, is an approximately eight meter long tube which digests food, absorbs nutrients and expels waste. The gut stretches from the mouth to the anus (Williams, 2012, p.133). The gut can be divided into several parts, comprising the stomach, small intestine, large intestine (also called the colon) and rectum, which is illustrated in Figure 1. In humans, the main digestion process is carried out in mouth, stomach, and small intestine. During this process, nutrients are made available for uptake. Nutrients are mainly taken up in the small intestine. In the large intestine, the microbiota ferment indigestible parts of the food such as dietary fibers. Simultaneously, water and salts are extracted and absorbed.

The intestine is divided into the small intestine and the colon also called the large intestine. The colon can further be divided into the distal and the proximal colon (Figure 1).

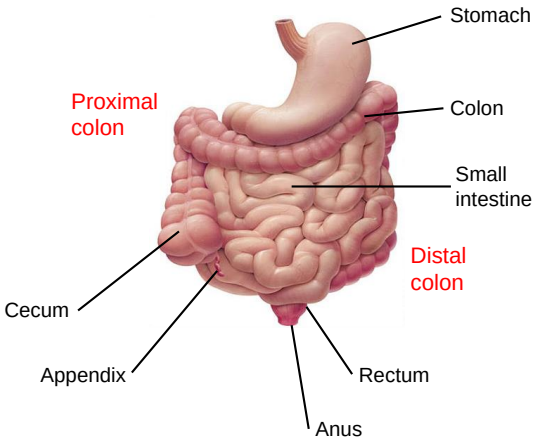


Figure 1: Sections of the human gastrointestinal tract (adapted from von der Burchard (2012) (24.4.2015)).

1.1.3 Colon cancer

One definition of cancer is that cells in the body divide without control and spread into the surrounding tissue. When the cells are dividing and an abnormal mass of tissue is formed this mass is called a tumor. Tumors can be malignant or benign. Benign tumors lack the ability to invade other tissues in the body. When benign tumors are left untreated they have the potential to become malignant. Malignant tumors can innvade the surrounding tissue and can spread into tissues further away. The process when a tumor is developing from a benign to a malignant form is accompanied by angiogenesis. In angiogensis, the formation and maturation of blood vessels occurs in order to provide the needed oxygen and nutrients to the growing tumor.

Cancer that occurs in the appendix, colon, or rectum is called colorectal cancer. The specific colorectal cancer types share many characteristics and are therefore often studied as a group. According to Stewart and Wild (2014), colorectal cancer was the fourth most frequent cancer type after lung, liver, and stomach cancer world-wide in 2014. Norway was one of the countries with the relatively highest occurrence of colorectal cancer in the time period 2009 to 2013 (Larsen, 2013). In a preliminary phase of colorectal cancer, normal colonic epithelium cells start dividing and forming benign polyps. In 80% of the cases these polyps are formed due to a mutation in the *adenomatous polypois coli* (*APC*) gene (Rowan et al., 2000). *APC* is a tumor suppressor gene that prevents the cell from uncontrolled growth. Polyps can progress into malignant growing carcinoma. This step is often accompanied by an alteration in the tumor suppressor gene *TP53* which encodes the protein p53. This protein plays an important role in inhibition of angiogenesis and apoptosis (the process of programmed cell death) and a mutation in the gene may result in the resistance of the

cell to apoptosis.

Colorectal cancer development is driven by the loss of genomic stability. This can either be chromosomal or microsatellite instability. When chromosomal instability occurs the chromosomal copy number and structure will be changed and the wild-type copy number of genes such as *APC* and *TP53* can get lost (Markowitz and Bertagnolli, 2009). In microsatellite instability, alterations in the length of microsatellite sequences will occur due to an inactivated DNA mismatch repair system. Mutations in coding sequences result in frameshift mutations and these can cause the production of truncated or functionally inactive proteins (Kim et al., 2013).

The development of cancer is frequently due to genetic mutations but these mutations are often not sufficient. Chronic inflammation is also associated with the development of cancer (Balkwill and Mantovani, 2001; Coussens and Werb, 2002). Chronic intestinal inflammation can cause inflammatory bowel disease. A direct link between chronic diseases and the development of cancer was observed among patients with inflammatory bowel disease. These patients had an increased risk of developing colorectal cancer (Zhang and Hamaker, 2010). In the cancer development, the inflammation level is high and there is an ongoing vascular remodeling and proliferation of tumor vessels (Nelson and Ganss, 2006). Furthermore, the tumor needs enzymes such as matrix metalloproteinases for growing and invading new tissue (Mook et al., 2004). Also the enzyme prostagladin-endoperoxide synthase 2, which is encoded by the gene *PTGS2*, is associated with the control of cell growth is over-expressed in colorectal tumor tissue (Eberhart et al., 1994).

1.1.4 Dietary fibers

Dietary fibers are a heterogeneous group of carbohydrate polymers. According to the CODEX Alimentarius Commission, dietary fibers are defined as carbohydrate polymers with ten or more monomeric units, which are not hydrolyzed by endogenous enzymes in the small intestine of humans. Dietary fibers can be naturally occurring in food as consumed, obtained from raw material or be synthetic carbohydrate polymers. Fibers obtained from raw material or synthetic carbohydrate polymers must show a proven physiological benefit in human health in order to be officially accepted as dietary fibers (World Cancer Reserach Fund / American Institute for Cancer Research, 2007).

Dietary fibers can be classified as either resistant starch (starch that resists digestion), non-starch polysaccharides, or lignin. Cellulose, pectin, arabinoxylan, and β-glucans belong amongst others to the group of non-starch polysaccharides. Dietary fibers can be soluble or insoluble in water. Soluble fibers, such as pectin, cereal β-glucan, and some arabinoxylans are fermented in the proximal colon. They can absorb water, form viscous solutions, and slow down the digestion.

Soluble fibers have the potential to lower blood cholesterol levels and regulate the blood glucose postprandial rise (Brennan and Cleary, 2005). Sources of soluble fibers can be oat, barley, carrots, and apples. Insoluble fibers, namely cellulose, lignin, some arabinoxylans, and β-glucans from yeast pass relatively intact through the gastrointestinal tract. They are fermented more slowly in the distal colon, rectum and faeces than soluble fibers, and they have a laxative effect and increase the faecal bulk (McIntyre et al., 1991). Whole wheat flour, nuts, green beans, and potatoes are good sources of insoluble fibers. Most foods contain both soluble and insoluble fiber.

1.1.5 Dietary fibers and colon cancer

General Development of colorectal cancer and diet are directly linked: it is estimated that 70% of all cases of colorectal cancer are provoked by dietary factors (Young et al., 2005). The consumption of red meat, fat, alcohol, and lifestyle issues such as smoking and obesity are associated with colorectal cancer (Qasim and O'Morain, 2010). Burkitt (1977) hypothesized that an increased intake of dietary fibers may reduce the occurrence of colon cancer. This hypothesis was based on observations of lower colon cancer rates in East Africa compared to more industrialized countries. The fiber-rich diet comprising unrefined grains and vegetables in Africa can be a reason for this observation. Burkitt (1969) assumed that dietary fibers can protect against colon cancer. This was not confirmed in later studies. The case control studies of Freudenheim et al. (1990) and Howe et al. (1992) support the assumption of Burkitt (1969) and showed a strong to moderate protective effect of dietary fibers against colon cancer. Both studies conclude that reducing the risk of developing cancer by 50% requires the consumption of approximately 30 g fibers every day. The source of fiber was not defined in these studies. In epidemiological studies, the protective effect of dietary fiber was not as strong as in the case control studies and only weak protective effects of dietary fiber could be verified (Kim, 2000). The different outcomes of the studies may be due to different fiber sources used. Fibers from different sources can differ profoundly in their structures as dietary fibers are a heterogeneous group comprising carbohydrates polymers with various structures that most likely these will have different effects (Aune et al., 2011). For determining which type of fibers have the greatest beneficial potential and for understanding their protective effects, the molecular mechanisms need to be studied.

Potential mechanisms behind the protective effect The mechanism behind the protective effect of dietary fibers against colorectal cancer is not fully evaluated. However, faecal bulking and bacterial fermentation are two of the most likely possible mechanisms.

Dietary fibers can bind water which increases the faecal bulk. This leads to a dilution of

carcinogens (Harris and Ferguson, 1993). Faecal bulking is also associated with a reduced transit time and a shorter time period in which epithelium cells in the colon and rectum are exposed to carcinogenic compounds (Ferguson et al., 2000).

The adult colon is the habitat of approximately 10^{14} bacteria from over 1000 different species (Zhang and Hamaker, 2010). These bacteria are the most abundant group of microorganisms that form the gut microbiota. The host organism and the microbiota form a mutualistic relationship. During the colonic fermentation of dietary fibers, the microbiota utilize food components that were not digested in the small intestine. This process serves as an energy source for the microbiota. Dietary fibers undergo fermentation. Short chain fatty acids (SCFA) are the most important metabolites that are formed during fermentation of dietary fibers. SCFA are energy substrates for the colonocytes (colonic epithelial cells), play a key role in colon health (Canani et al., 2011), and have anti-inflammatory properties (Jacobasch et al., 1999). Due to the heterogeneity of dietary fibers, different microorganisms will have the ability to utilize different types of dietary fiber. The source of dietary fibers will therefore drive a selection towards the microorganisms with best ability to digest the typical type of fibers consumed and the amount of these microorganisms will increase. Changes in the fiber composition leads to alterations in the microbiota and the composition of SCFA.

Butyrate Beside acetate and propionate, butyrate is the main SCFA produced in dietary fiber fermentation. Besides its effect as epithelium energy source, it also induces apoptosis when added to carcinoma cell lines *in vitro* (Hinnebusch et al., 2002). At the same time, butyrate acts as an anti-inflammatory agent through the inhibition of the activation of the inflammation associated nuclear factor kappa B (NF-κB) (Segain et al., 2000). Furthermore is the over-expression of the enzyme prostaglandin H2 synthase 2 (PTGS2) in colon tumor tissue suppressed by butyrate (Tong et al., 2004). The inhibition may be due to butyrate's ability to inhibit the enzyme histone deacetylases.

Beta-glucans A heterogeneous group of dietary fibers are β-glucans, consisting of β-linked glucose monomers. They are structural components of the cell wall in fungi, yeast, some bacteria, and certain grains. Their structure differs depending on the source. Cereal β-glucans, for example, are linear molecules consisting of mixed linked β-(1,4)-β-(1,3) glucose monomers (Figure 2). They are primarily found in barley and oat and are largely soluble in water. Yeast β-glucans on the other hand, are branched molecules consisting of a backbone of β-(1,3)-linked glucose monomers with β-(1,6)-linked side chains (Figure 3). Most yeast β-glucans are insoluble in water. Cereal and yeast β-glucan have been ascribed immune-modulating properties and several studies have reported

anti-tumor or immune-modulating effects (Ross et al., 1999; Lee et al., 2001; Davis et al., 2004; Qi et al., 2011; Stier et al., 2014). Oral administration of yeast and barley β-glucans has, for example, been shown to increase the efficacy of anti-tumor monoclonal antibodies in a mouse tumor model (Hong et al., 2004). The effect could be related to the carbohydrate structure (Hong et al., 2004). Yeast β-glucans are often found to have a more pronounced effect than cereal β-glucans. The direct structure-dependent immune-modulating potential of certain dietary fibers is increasingly recognized (Wismar et al., 2010). In this thesis, a particulate yeast β-glucan preparation of high purity denoted dispersible wellmune was therefore chosen as a representative of this group of fibers with a potential immune-modulating effect.

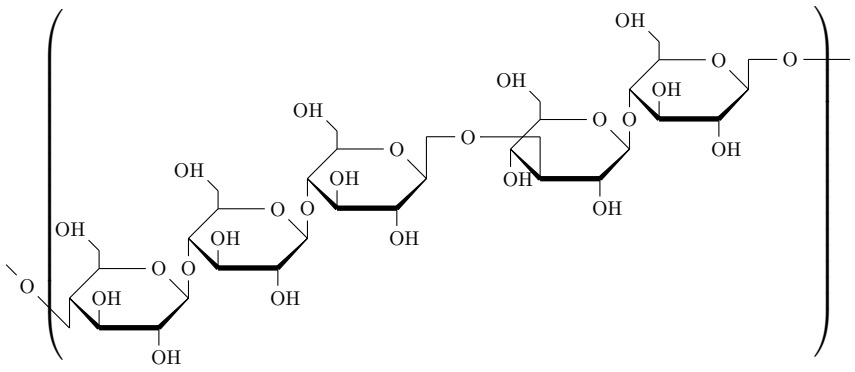


Figure 2: Structure of cereal β-glucan. Figure adapted from Volman et al. (2008)

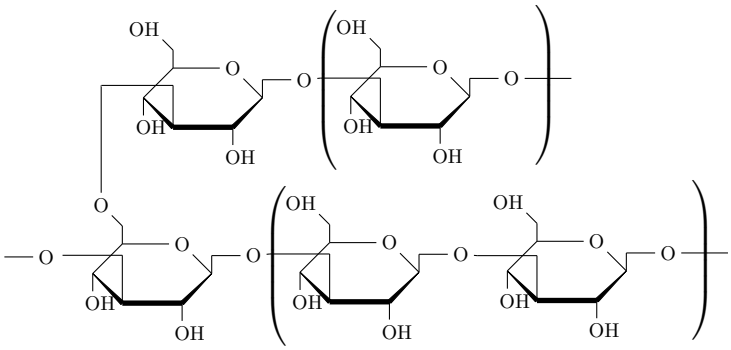


Figure 3: Structure of yeast β-glucan. Figure adapted from Volman et al. (2008)

1.2 Background: Quantitative real-time reverse transcription PCR

1.2.1 Gene expression

For getting a better understanding of the molecular effects of dietary fibers, the expression of specific genes can be studied. In gene expression, the nucleotide sequence of a gene is used for synthesis of a functional gene product. Quantitative real-time reverse transcription PCR (RT-qPCR) became an extensively applied technique for quantitative analysis of gene expression.

1.2.2 General introduction to RT-qPCR

Since its invention by Mullis in 1983 (Mullis et al., 1990), the polymerase chain reaction (PCR) became a laboratory technique of greatest impact in biological research. With PCR it became possible to amplify specific regions of DNA sequences. PCR is a common molecular biological application and is often used in gene expression analysis, DNA cloning, and DNA sequencing. For determining whether the target DNA sequence was amplified or not, downstream processing like gel electrophoresis is needed. In order to improve the PCR, Higuchi et al. (1992) omitted the downstream processing by adding ethidium bromide to the PCR. Ethidium bromide binds to double-stranded DNA and its fluorescence increases with the increasing amount of amplified DNA enabling real time monitoring of the PCR.

Higuchi et al. (1992) accomplished the first demonstration of real-time PCR (qPCR). The qPCR refers to PCR amplified DNA that can be monitored and measured after each PCR cycle. In qPCR, fluorogenic probes are used that make it possible to detect a fluorescence signal after each PCR cycle.

RNA cannot be amplified in PCR and a DNA template is needed. RNA can be converted into DNA by reverse transcription. Reverse transcription is originally a process used by retroviruses where the enzyme reverse transcriptase synthesizes complementary DNA (cDNA) in the host cell from virus RNA. In this way, retroviruses incorporate their genetic material into the host genome. Since the first description of the reverse transcriptase by Howard and Satoshi (1970) and Baltimore (1970) the method is used in molecular biological processes such as creating cDNA libraries and PCR based applications. A reverse transcription reaction followed by PCR is called reverse transcription PCR (RT-PCR). When qPCR and RT-PCR are combined, this process is called real-time RT-PCR (RT-qPCR). RT-qPCR comprises of the following steps: (i) RNA isolation, (ii) cDNA synthesis, and (iii) PCR.

1.2.3 Different steps in RT-qPCR

cDNA synthesis By combining the reverse transcription reaction with the PCR it becomes possible to study RNA transcripts with a low copy number of starting material (Dorak, 2006, p.4). In RT-PCR and RT-qPCR single-stranded cDNA templates are synthesized for later PCR. This is called first-strand cDNA synthesis. In the first-strand cDNA synthesis in RT-qPCR, a single stranded DNA primer is hybridized to a single stranded RNA template. Reverse transcriptase then binds to the primer:RNA template complex and starts the reverse transcription. The reverse transcriptase has three different activities: (i) RNA-dependent DNA polymerase, (ii) ribonucle-

ase H, and (iii) DNA-dependent DNA polymerase activity. For the cDNA synthesis in RT-qPCR, activity (i) and (ii) are utilized. Today, the two most commonly used reverse transcriptases for cDNA synthesis are the reverse transcriptases of the Avian myeloblastosis virus (AMW) and the Moloney murine leukemia virus (MMLV) (Okello et al., 2010). When the reverse transcriptase is bound to the primer:RNA template complex, it starts the DNA synthesis from the 3' end of the DNA primer and adds free nucleotides to the 3' end of the new formed strand. The RNA strand serves as template for this reaction. A DNA:RNA hybrid is formed. The RNase H activity of the reverse transcriptase may lead to the degradation of RNA in the DNA:RNA hybrid. However both DNA:RNA hybrid and single stranded DNA can be used as staring material for the following PCR as the initial denaturating step will break up all double-stranded DNA:RNA hybrids.

The right choice of primer in cDNA synthesis is essential for the efficiency of this step. There are three different types of primers that can be used for cDNA synthesis: (i) sequence specific, (ii) oligo(dT), and (iii) random hexamer primers.

Sequence specific primers bind only to a specific sequence on the mRNA. These primers offer high specificity. At the same time they are not very flexible since a new cDNA synthesis has to be performed for each gene included in the study.

Oligo(dT) primers are more flexible than sequence specific primers since they do not bind to a specific sequence but more generally to the poly(A) tails of messenger RNA (mRNA) molecules (Figure 4A). This allows the study of several genes in the ensuing PCR amplification. A disadvantage using oligo(dT) primers is that they are biased towards the 3' end of the transcript. Because the reverse transcriptase enzyme sometimes falls off and the 5' end is missing, it can be difficult to produce full length cDNA (Dorak, 2006, p.4).

Random hexamer primers are six nucleotides long. The shortness of these type of primers open for many possible binding sites in the target. They are therefore thought to bind "randomly" to any site of the target RNA template (Figure 4B). In contrast to oligo(dT) primers, they can bind also to non-polyadenylated RNA. Random hexamer primers have the disadvantage to be biased towards the 5' end of the target (Dorak, 2006, p.4). Since all RNA is

In summary, each primer type has advantages and disadvantages. Before starting a new RT-qPCR experiment the primer choice should be evaluated towards the primer with best sensitivity and accuracy in the cDNA synthesis.

PCR In a PCR, a DNA template is copied. Each PCR cycle consists of three steps: (i) denaturation, (ii) annealing, and (iii) elongation. In the denaturation step, double-stranded DNA or cDNA:RNA hybrid templates are denaturated at approximately 95 °C. The annealing step is ini-

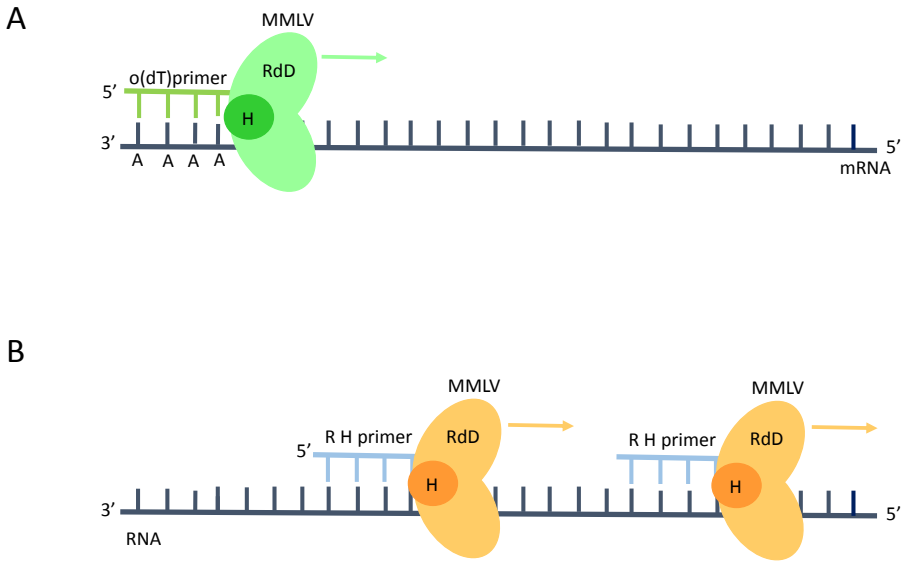


Figure 4: The use of oligo(dT) and random hexamer primers in cDNA synthesis. The reverse transcriptase of MMLV binds to the specific primer. With its RNA dependent DNA polymerase activity (RdD), the polymerase generates cDNA from the RNA template. After cDNA synthesis, the RNA can be degraded by the ribonuclease H (H) activity of the reverse transcriptase. **A:** The primer oligo(dT) (o(dT)) anneals to the poly A tail of the mRNA strand. **B:** Two binding possibilities of the random hexamer (R H primer) primer annealing to the RNA strand.

tiated by lowering the temperature to approximately 55 °C such that primer and probe can anneal to the single-stranded DNA. The annealing temperature depends on the primer sequence. In the elongation step, the temperature is increased to 72 °C which is the optimal temperature for DNA polymerase activity. DNA polymerase extends the primers by adding complementary nucleotides to the DNA template. This process is repeated 25 to 40 times (McPherson and Møller, 2006).

DNA polymerases used in PCR need to be heat resistant. The two most commonly used DNA polymerases are *Pfu* and *Taq* DNA polymerases (Valasek and Repa, 2005). *Taq* polymerase is the DNA polymerase of the thermophilic gram-negative bacterium *Thermus aquaticus*. *Taq* polymerase has two catalytic activities, one domain for synthesizing a new DNA strand and a 5'-nuclease domain for cleaving DNA bound downstream of the DNA synthesis.

Real-time PCR uses the same mechanisms as conventional PCR, with the addition of a fluorescent agent which enables the monitoring of DNA sequence amplification in real-time. Two major principles can be distinguished: (i) fluorescent dyes that bind to double-stranded DNA and (ii) dye-labeled sequence specific probes, that bind to single-stranded DNA. Only the latter will be further described.

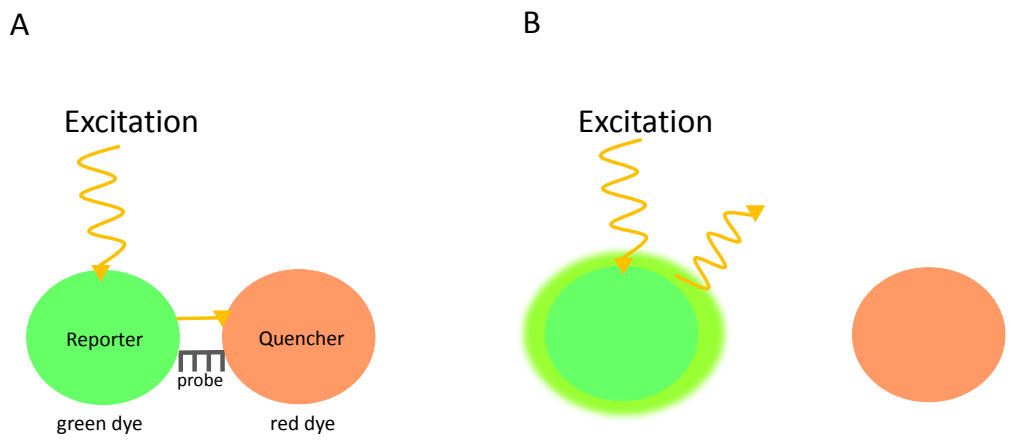


Figure 5: The FRET mechanism. In this example the green dye is the reporter dye and the red dye is the quencher dye. **A:** The green and the red dye are bound to the same probe. After excitation of the green dye, FRET occurs and the emission energy is transferred from the green dye to the red dye. **B:** FRET cannot occur because of the large distance between green and red dye. The green dye emits its signal. (Adapted from: Life Technologies (2012))

1.2.4 TaqMan – a representative of dye-labeled sequence specific probes

Dye-labeled sequence specific probe chemistry like TaqMan (Thermo Fisher Scientific, Waltham, Massachusetts, USA) utilizes the fluorescence resonance energy transfer (FRET) mechanism. In this mechanism the emission of one fluorescent dye is reduced by the close proximity of another dye bound to the same probe. The mechanism of FRET is exemplified in Figure 5A. The green dye is excited by light. When the green and the red dye are close to each other, FRET will occur. Instead of emitting the light to return from the electronic excited state to ground state, the green dye transfers the energy directly to the red dye. In other words, the red dye quenches the green dye. Since the efficiency of the energy transfer is highly dependent on distance, FRET is negligible when the two dyes are not in close proximity. In this case, the green dye returns to the ground state by emitting light (Figure 5B) (Life Technologies, 2012).

As mentioned above, TaqMan assays consist of sequence-specific probes and two sequence-specific primers. The probe is fluorescently labeled by a quencher at the 3' end and a reporter at the 5' end of the probe. FRET occurs when reporter and quencher are in close proximity. Different reporter and quencher dyes can be chosen. Here only the TAMRA quencher and the MGB probe will be mentioned.

TAMRA is a quencher used since a long time. It is a fluorescent molecule in itself and gives therefore a weak background signal (Life Technologies, 2012). TAMRA probes tend to be long because the melting temperature of the probes have to be higher than the melting temperature

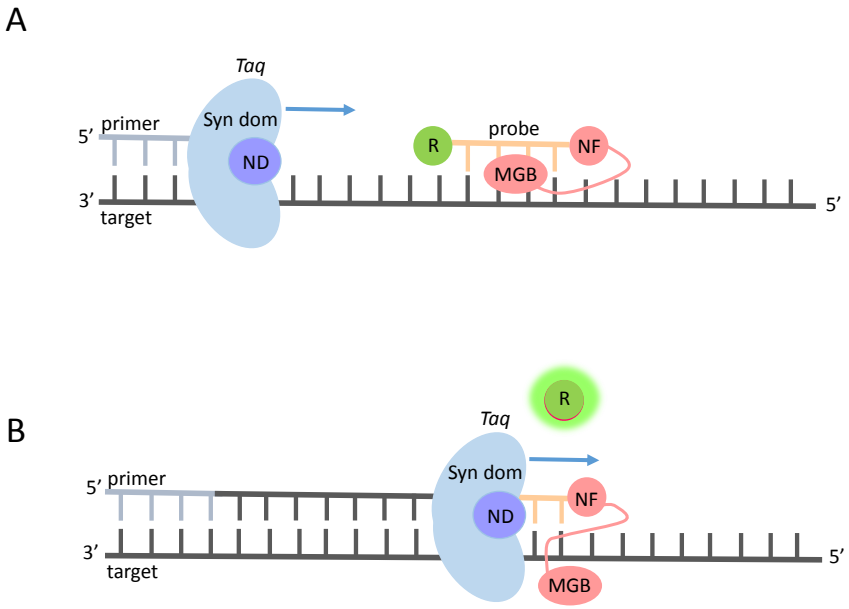


Figure 6: Use of TaqMan chemistry in PCR. **A:** The TaqMan probe consists of a reporter (R) and a non-fluorescent quencher (NF) bound to a minor groove binding (MGB) molecule. Taq DNA polymerase (*Taq*) consists of a synthesis domain (Syn dom) and a 5' nuclease domain (ND). Taq DNA polymerase synthesizes DNA in 5' - 3' direction (indicated by a blue arrow). **B:** During PCR, *Taq* displaces the probe and cleaves the reporter. FRET does not occur any longer and the reporter fluoresces.

of the primers. This way, it is ensured that the probes bind strongly enough before the primers anneal and the DNA synthesis starts. But long probes can give lower specificity. To overcome this problem it can be advisable to use shorter probes, like MGB probes (Figure 6) (Life Technologies, 2012).

At the 5' end of the MGB probes, a reporter dye is bound while at 3' end of the MGB probes two subunits are bound: (i) a non- fluorescent dye (NFG) and (ii) a minor groove binding (MGB) molecule which is attached to NFG. NFG gives no background fluorescence. A minor groove is formed when the probes bind to the target sequence. MGB can bind to this groove and strengthen the probe:target sequence binding. In this case, it is possible to generate shorter probes (Life Technologies, 2012).

Before a thermostable DNA polymerase, such as *Taq* polymerase starts DNA synthesis, only small amounts of quenched reporter dye can be measured, this is independent of the chosen quencher dye (Figure 6A). *Taq* polymerase has two domains, the synthesis domain, responsible for the DNA synthesis and the 5' nuclease domain responsible for degradation of downstream DNA. *Taq* polymerase synthesizes DNA in 5' - 3' direction. When it reaches the probe at its 5' end, the reporter is cleaved off by the 5' $\rightarrow$ 3' exonuclease activity of the polymerase. Reporter

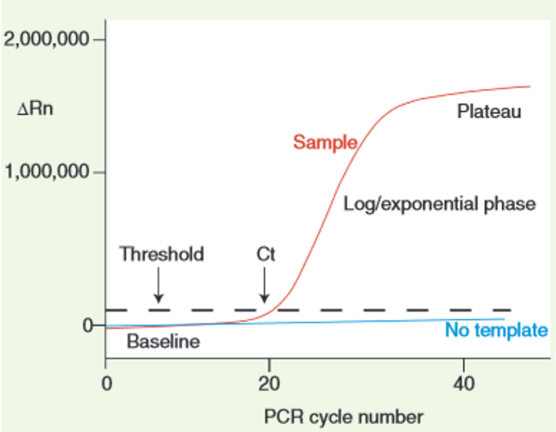


Figure 7: Schematic graph of a single amplification plot in RT-qPCR. C_T denotes the threshold cycle and ΔRn the fluorescence signal of the amplification product subtracted from the fluorescence signal of the baseline (adapted from Arya et al. (2005)).

and quencher are permanently separated, consequently FRET cannot occur any longer, and the reporter dye fluoresces. The resulting fluorescence signal is proportional to the amount of amplified sample. *Taq* polymerase continues the DNA synthesis.

The application fluorescent probes like TaqMan result in an increase in florescence by a successful amplification. These fluorescence signals can be detected and visualized by amplification plots as illustrated in Figure 7.

ΔR_n is the baseline corrected fluorescent signal and is calculated by

$$\Delta R_n = R_n f - R_n b, \tag{1}$$

where $R_n f$ is the amount of fluorescence signal of the PCR product at each cycle and $R_n b$ is the amount of fluorescence signal at the baseline.

The baseline of RT-qPCR is defined as the initial PCR cycles where only little changes in the fluorescence signal occur (Dorak, 2006, p.xix). This level of fluorescence is the background of the reaction.

The threshold is the level where the fluorescent signal of the RT-qPCR increases significantly. The threshold distinguishes the background from the relevant amplification. It is calculated as ten times the standard deviation of the baseline fluorescent signal (Arya et al., 2005).

The threshold cycle (C_T) is defined as the number of PCR cycles needed for the fluorescent signal to cross the threshold (Walker, 2002). The C_T is inversely related to the starting amount of template in the reaction. Increasing the amount of template DNA in the beginning of the reaction leads to smaller C_T values. When the reaction components become limited, the amplification rate will decrease. This phase is called the plateau phase.

The C_T values can reach a threshold after which the detection of the gene expression is considered as unreliable. For the TaqMan assay this threshold is for example everything above 35 cycles. A C_T value above this may correspond to a gene which is not or only expressed in a small amounts (Life Technologies, 2010).

1.2.5 Absolute and relative quantification

Absolute quantification RT-qPCR can quantify gene expression by absolute or relative quantification. The absolute quantification is used when the expression level of absolute numbers needs to be determined such as the number of viral particles per ml of blood (Bio-Rad Laboratories, 2006). The quantity of a target gene is estimated from a range of standards with known concentrations. The standard with known quantity is serially diluted and a standard curve is generated by plotting the log quantity against the C_T values. By interpolation, the quantity of the unknown target can be measured (Bio-Rad Laboratories, 2006). For absolute quantification it is essential to have stable and reliable standards with known amounts of target genes.

Relative quantification One way to quantify relative changes in target gene expression is by setting the change in relation to at least one reference. Using reference genes is the most common method for normalization of gene expression (Bustin et al., 2009).

In normalization, different amounts of RNA starting material, and variations in reverse transcription yield can be controlled. Since RT-qPCR comprises further on a lot of processing and extraction steps, a proper normalization step is of high importance to get sensitive and reliable results.

The suitability of the reference genes has to be validated for a particular cell type or tissue (Bustin et al., 2009). A good reference gene should fulfill four criteria: it (i) needs a stable expression in different experimental treatments and developmental stages, (ii) has a similar amplification efficiency as the target gene, (iii) should be abundantly expressed, and (iv) should be expressed at the same level as the target gene (Ling et al., 2014). Programs such as NormFinder, BestKeeper or geNorm can be used to select the most stable reference genes after an amplification reaction. All these programs are based on different algorithms and this can result in different suitabilities of the reference genes (Anstaett et al., 2010). It is also advisable to use more than one stable reference gene for normalization. According to Bustin et al. (2009), the use of a single reference gene for normalization is not acceptable unless clear evidences for the use of single reference genes exist (e.g. previously tested and validated reference genes for equal experiments and conditions). Vandesompele et al. (2002) showed that the use of single reference genes lead to erroneous nor-

malization in 25% of their tested samples. Ideally, reference gene selection should be performed by testing the stability of reference genes after three independent measurements (biological parallels) with three technical parallels each, using three different validation programs. This is also known as the rule of "Best 3" (Kozera and Rapacz, 2013).

Reference genes One of the most commonly used reference genes for the normalization process is glyceraldedyde 3-phosphate dehydrogenase (*GAPDH*). For a long time it was assumed that *GAPDH* is only involved in glycolysis. However, in the early 2000s it was found that *GAPDH* is, among others, involved in both transcriptional and post-transcriptional gene regulation (McKnight, 2003; Bonafe et al., 2005). The opinions differ between researchers if *GAPDH* is useful as reference gene or not. While Edwards and Denhardt (1985) and Winer et al. (1999) showed good results in their studies using *GAPDH*, Barber et al. (2005) and Bustin (2000) did not recommend it as reference gene. As Barber et al. (2005) verify in their study, the mRNA expression level for *GAPDH* can differ significantly between different human tissues while the expression level tends to be more similar within related groups of tissues like duodenum, jejunum, ileum, and colon.

Another commonly used group of genes for normalization are genes encoding ribosomal subunits, with *18S rRNA* as a well-known representative. As much as 85% to 90% of the total RNA in a cell constitutes rRNA (Bustin, 2000). As with the use of *GAPDH* as a reference gene, it is important to ensure that the expression of *18S rRNA* is not affected by the particular experimental treatment. Bas et al. (2004) analyzed the expression of different genes of T-lymphocytes. They identified *18S rRNA* as a reference gene that is expressed in stable levels at different activation stages. However, Fu et al. (2010) tried to determine genes for normalization in ovarian tissue and found that *18S rRNA* was unsuitable as a reference gene. Furthermore, there are some other drawbacks that need to be taken into consideration when rRNA is used as a reference gene. In contrast to mRNA, ribosomal subunits are not polyadenylated. As a result of this, no oligo(dT) primer can be used in cDNA synthesis. Furthermore, when using rRNA as reference, total RNA must be used in the RT-qPCR and not purified mRNA fractions since rRNA is eliminated from these samples. Finally, rRNA can be expressed in greater levels than the target gene. This is in conflict with the requirements for reference genes mentioned above.

1.3 Test system description

In the thesis, a test system was established to study the protective effects of dietary fibers and their metabolites in colon cancer by using RT-qPCR. In the following paragraphs, the components of the test system besides the potential reference genes *GAPDH*, *18S rRNA*, β_2-microglobulin (*B2M*), and beta-actin (*ACTB*) will be presented.

Cell lines HT-29 and HCT-116 are both human colon carcinoma cell lines. According to the American Type Culture Collection, the provider of the cell lines, HT-29 cells have a point mutation in the *TP53* gene. In contrast to HT-29 cells, the *TP53* gene is of wild type form in HCT-116 cells. The cell lines serve as two different models for colon cancer in the thesis. In order to have more control over the test system, cell lines were chosen instead of tissue samples. Cell lines provide a pure population which is not a mixture of different cell types as it is the case in tissue samples (Kaur and Dufour, 2012) and enables reproducible results.

Target genes The target gene *NFκB1* encodes the protein nuclear factor kappa-B p105 subunit (NFκB1). NFκB1 is one of the structurally related proteins in the NF-κB transcription factor family. The NF-κB transcription factor family regulates the expression of genes involved in immune and inflammatory responses (Karin et al., 2004). At the same time NF-κB also regulates the expression of genes which are associated with cancer development and progression (Dolcet et al., 2005). Lind et al. (2001) found that NFκB was up-regulated in human colorectal cancer and they further suggested that an inhibition of NFκB may be useful in anti-tumor therapy.

The target gene *PTGS2* encodes the human enzyme prostaglandin-endoperoxide synthase 2 (PTGS2). PTGS2 is expressed during inflammation and produces prostaglandins (Parrett et al., 1997). Prostaglandins stimulate angiogenesis and inhibit immune surveillance and are therefore associated in promoting tumorgenesis (Ben-Av et al., 1995). Eberhart et al. (1994) showed that PTGS2 is over-expressed in colorectal cancer.

The gene *NFE2L2* encodes the nuclear factor erythroid 2-related factor 2 (NFE2L2). The transcription factor NFE2L2 can be activated due to oxidative stress which is an imbalance between the production of free radicals and reactive metabolites and the detoxification of these reactive metabolites (Reuter et al., 2010). NFE2L2 regulates genes with antioxidant response elements (ARE) in their promotor. Most of these genes are involved in stress-response mechanism and protection of cells against carcinogens. An increased expression of ARE regulated genes is linked to inhibition of the development of cancer (Zhang and Gordon, 2004).

Treatment types Butyrate is one of the most abundant SCFA produced by bacterial fermentation in the colon and has a reported anti-carcinogenic effect. Butyrate us innvolved in the regulation of genes associated with cell proliferation and apoptosis (Gonçalves et al., 2011). In the human colon, butyrate concentrations vary between 5 mM (Treem et al., 1994) and 24 mM (Cummings et al., 1987).

Dispersible wellmune is a food supplement product containing 84% refined yeast-derived β-(1,3)/(1,6)-glucans. Furthermore the product consists of 7% water and 6% fat fraction. Dispersible wellmune is known to have an immune-modulatory effect. Fuller et al. (2012) showed that dispersible wellmune had a tendency to decrease cold and flu symptoms. Talbott et al. (2013) found an improvement of allergy symptoms for ragweed-allergy sufferers after four weeks with dispersible wellmune treatment.

1.4 Aim of the thesis

The main aim of this thesis was to establish a test system to study the protective effect of dietary fibers and their metabolites against colon cancer. The test system is supposed to give new insights into the molecular mechanisms of this protective effect and at the same time enable the selection of dietary fiber types or metabolites with the highest protective potential in future studies. In order to achieve this, the commonly used RT-qPCR technique was adapted for the use in colon carcinoma cell lines HT-29 and HCT-116. The yeast-derived β-glucan product dispersible wellmune and the dietary fiber metabolite butyrate were used as model substances to evaluate the test system. The following specific aims were defined:

1. Adaption of the RT-qPCR method which included the development of optimally adjusted RNA isolation, cDNA synthesis, and PCR methods;
2. Examination and selection of stable reference genes for the RT-qPCR method;
3. Selection of an appropriate butyrate concentration;
4. Evaluation of the suitability of the established test system and examination of changes in target gene expression in human colon carcinoma cell lines due to the exposure to dispersible wellmune and butyrate.

2 Material and Methods

2.1 Cell cultures

Theory: Cell cultures Cell cultures are frequently used tools in biological research. They can, among others be, used in toxicity testing of new drugs or in cancer research to study differences between normal and cancer cells, or different cancer treatments (Cree, 2011). Cell cultures can be obtained from tumor cells.

When initial or primary cell cultures are passaged several times, a cell line is produced. The advantage of using cell lines as a model system in studies, is the ability to provide renewable material for repeatable studies since subculturing of cell lines results in a quite homogenous populations of cells. Tumor-derived cell cultures can be passaged many times when the cells are maintained at appropriate conditions. Growing cells require amino acids, vitamins, metal ions, and an energy source. While glucose is often the energy source, the other missing substances are typically provided by cell culture media and serum. The most widely cell culture media today are Eagle's minimum essential medium (MEM) and its modification Dulbecco modified MEM (DMEM) together with Roswell Park Memorial Institute (RPMI) medium. Fetal bovine serium (FBS) is frequently used as serum. To protect cell lines from or to treat cell lines with bacterial infections, antibiotics like streptomycin and penicillin are often added to the cell culture medium.

In this study the three human colon carcinoma cell lines HT-29, HCT-116, and Caco-2 where used.

Application: Cells, reagents and general culture conditions The human colon carcinoma cell lines HT-29 and Caco-2 were a generous gift from Professor Tor Lea, Norwegian University of Life Sciences. The human colon carcinoma cell-line HCT-116 was a generous gift from Professor Gunhild Mælandsmo, Oslo University Hospital. All three cell lines were originally obtained from the American Type Culture Collection (ATCC). HT-29, Caco-2 and HCT-116 cells were cultured in DMEM (Thermo Fisher Scientific, Waltham, Massachusetts, USA) supplemented with 10% FBS (Thermo Fisher Scientific), 1% non-essential amino acids (NEAA), 100 U ml^{-1} penicillin, and 100 µg mL^{-1} streptomycin. All cultivations (subculturing and treatment) were carried out at 37 °C with 5% CO_2 in a humidified atmosphere.

Application: Subculturing of cell lines For subculturing of HT-29, Caco-2 and HCT-116 cells, the culture medium was removed and the cells were washed twice with 5 mL phosphate-buffered saline (PBS) (Thermo Fisher Scientific). To detach the adherent cells from the cell culture

flasks (Sigma-Aldrich Co, St. Louis, Missouri, USA), 3 mL trypsin-EDTA (1X) (Thermo Fisher Scientific) were added and incubated at 37 °C. After the detachment of the cells, the trypsinization reaction was stopped by adding 8 ml cell culture medium and mixing the solutions properly. An appropriate amount of cell suspension was added to fresh 37 °C preheated cell culture medium and incubated at 37 °C.

HT-29, Caco-2 and HCT-116 cells were subcultured twice a week. To avoid changes in cell line characteristics, only passages between five and 25 were used for experiments. Every sixth week the cells were tested for *Mycoplasma* infections using the MycoAlert™ Mycoplasma Detection Kit (Lonza, Basel, Switzerland).

Application: Cell treatment for MTT assay Each of the three cell lines, HT-29, Caco-2 and HCT-116, were seeded at a concentration of $2.0 \cdot 10^5$. The cell lines were seeded in 96-well plates with 100 µL cell culture medium and incubated for 24 hours. Only cells with a confluency between 70% to 90% were selected for experiments. After the first 24 hours of incubation, the medium was changed to fresh culture medium, or medium containing 0.5, 1.0, 2.5, 5.0, 7.5, 10.0, or 20.0 mM butyrate (Sigma Aldrich,Co, St. Louis, Missouri, USA) and cells were incubated for further 24 hours. All experiments were repeated at least three times and cell treatments were performed in triplicates.

Application: Cell treatment for RNA isolation HT-29 and HCT-116 cells were cultured in 12-well cell culture plates at a concentration of $2.0 \cdot 10^5$ ml^{-1} and incubated for three, six, or 24 hours. After this period the cells were treated with either fresh cell culture medium or cell culture medium containing 2.5 mM butyrate or 1 mg mL^{-1} dispersible wellmune (Biothera, Eagan, Minnesota, USA, Lot.No: 011). For the use in cell cultures, dispersible wellmune was suspended in Milli Q water and boiled for 30 minutes. Afterward, the cooled suspension was aliquoted in endotoxin free centrifuge tubes and freeze dried. The freeze dried dispersible wellmune aliquots were re-suspended in cell culture medium at a concentration of 1 mg mL^{-1} in all cell experiments.

The cell culturing for all experiments presented in this thesis, took a total of twelve month.

An overview of all replicates for the following gene expression analysis is shown in Table 1.

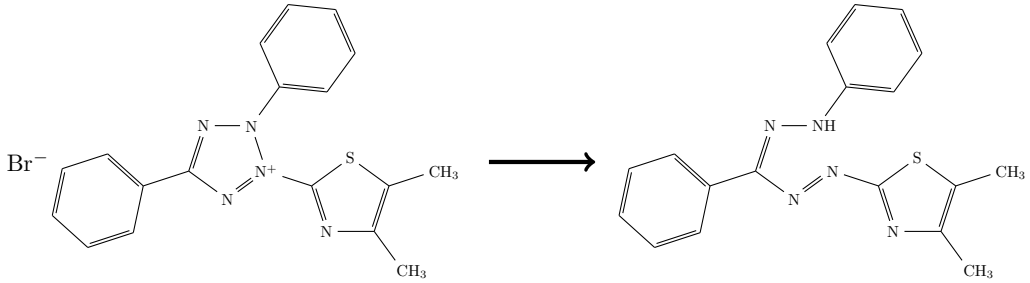
2.2 MTT assay

Theory There are various assays available for measuring cell viability. Until the 1980s most assays were based on the capacity of cells to incorporate radioactive substrate (e.g., Storr and Burton, 1974; Ashkenazi-Ezra and Ashkenazi, 1976) or to release a radioactive marker (e.g., Kromann

Table 1: Summary of replicates in gene expression analysis.

Cell line	Incubation time (hours)	Number of experimental replicates	Treatment replicates	Techniqual paralells in PCR
HT-29	3	1	2	2
	6	1	2	2
	24	3	2	2-3
HCT116	6	2	2	2

et al., 1980). An alternative for the radioactive based methods was described by Mosmann (1983). This assay is based on the ability of metabolically active cells to reduce the yellow tetrazolium salt MTT into purple formazan crystals (Figure 8). The formazan crystals can be dissolved in organic solvents and the resulting colored solution can be quantified using a spectrophotometer. The advantages of this assay are its high degree of accuracy and its ability to rapidly process large numbers of samples (Gerlier and Thomasset, 1986).



MTT Formazan

Figure 8: Principle of MTT assay. In the reaction the yellow MTT salt is reduced to purple formazan crystals.

Application In this thesis, the MTT assay was used to determine a butyrate concentration which was high enough to cause alterations in the gene expression of the cell line but low enough to avoid apoptosis. The treated and untreated cells were exposed to 15 µL MTT solution (Roche Applied Science, Penzberg, Germany). The cells were incubated for 2 hours at 37 °C after which the medium was removed. The formazan crystals were dissolved in 100 µL isopropanol containing 0.04 M HCl and the absorbance was measured at 562 nm using a microplate reader (SPECTROstar nano, BMG Labtech, Ortenberg, Germany). All experimental treatments were performed in triplicates and all experiments were performed at least three times.

2.3 RNA isolation

Theory RNA isolation is the process of extracting RNA from cells. Once extracted, RNA is very unstable and has only a short half-life due to the ubiquitous presence of RNases. For obtaining RNA of good quality, which is important for RT-qPCR experiments, RNA isolation relies on a sterile laboratory technique and RNase-free equipment. Additionally, isolated RNA samples should not be contaminated with genomic DNA or RNA-isolation reagents like alcohols and phenols because these can interfere with the RT-qPCR assay. To remove genomic DNA contaminations, a DNase treatment can be used. RNA concentration and purity can be estimated using an UV-Vis spectrophotometers such as NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific). To assess the purity of RNA, the NanoDrop spectrophotometer measures the ratio of absorbance at 260 nm and 280 nm. A value of approximately 2.0 indicates a pure RNA sample. A secondary measure of nucleic acid purity is the ratio of absorbance at 260 nm and 230 nm. For pure RNA samples, the value should be between 2.0 and 2.2 (Thermo Fisher Scientific, 2008).

Application Total RNA of HT-29 and HCT-116 cells was extracted using the RNeasy Kit (Qiagen, Venlo, Netherlands). The cells were lysed by adding 350 µL of a guanidine-thiocyanate containing buffer. This buffer inactivates RNases and is still present when the cells are homogenized using QIA shredder spin columns (Qiagen). Ethanol is added to provide suitable binding conditions for the homogenate to bind to the membrane of the RNeasy Spin Column (Qiagen). Genomic DNA contaminations were removed using RNase free DNase Kit (Qiagen) according to the manufacturer's instructions. Other contaminants were washed away in several washing steps with the buffer solutions included in the RNeasy Kit and following the manufacturer's instructions. RNA was eluted with 30 µL RNase-free water and additionally diluted with 30 µL RNase-free water. The RNA concentration and purity was measured with a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific).

2.4 RT-qPCR

2.4.1 cDNA synthesis

Theory RNA is not stable as template in RT-qPCR. Therefore it has to be converted into cDNA using an enzyme called reverse transcriptase. At 25 °C, a single stranded DNA primer is hybridized to a single stranded RNA template. Most RNA targets are reverse transcribed into cDNA at 37 °C to 48 °C, as this is the optimal temperature range of the reverse transcriptase. At 85 °C, the reverse transcriptase is inactivated, thus preventing it from inhibiting the later PCR. The result of the

first-strand cDNA synthesis is a DNA:RNA hybrid or a single-stranded cDNA.

Application A fixed amount of the isolated RNA samples was used for synthesizing first-strand cDNA with TaqMan Reverse Transcription Reagents (Thermo Fisher Scientific, Waltham, Massachusetts, USA). Each RNA sample was uniquely diluted with RNase-free water to reverse transcribe 100 ng RNA into cDNA. Calculations are based on concentrations obtained from NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific). A mastermix containing all the reaction components, except the RNA, was prepared to ensure equal quality in all reverse transcription reactions. Control samples without the reverse transcriptase MultiScribe (a recombinant reverse transcriptase of MMLV) were included for each set of reactions (NRT samples). The 10 µL samples contained 1.0 µL RT-buffer, 2.2 µL $MgCl_2$, 2.0 µL deoxy dNTPs, 0.5 µL random hexamers, 0.2 µL RNase inhibitor, 0.25 µL MultiScribe or RNase-free water and 3.85 µL appropriately diluted RNA (to yield 100 ng total RNA). The reverse transcription reaction was performed in a PCR instrument (GeneAmp PCR system 9700, Thermo Fisher Scientific) with the following temperature settings: (i) 25 °C for 10 minutes, 48 °C for 5 minutes, 95 °C for 5 minutes and (ii) a cooling down-step to 4 °C. The cDNA was diluted with 90 µL RNase-free water to a reaction volume of 100 µL and stored at −20 °C until later use. All experiments were performed at least three times.

2.4.2 PCR

Theroy For amplifying the cDNA in PCR, the following temperature steps are recommended when fluorescent probes are used. The reaction starts with a 10 minutes activation step at 95 °C. This step is followed by a denaturation step at the same temperature for 15 seconds. RNA:DNA hybrids or newly formed DNA is melted at that temperature and serve as templates for the next PCR cycle. Annealing and extension are, in contrast to the procedure described on page 8, both carried out at approximately 60 °C. According to Nolan et al. (2006), this temperature is suboptimal for the amplification by *Taq* polymerase but at the same time it ensures more efficient cleavage of the probe.

Application The expression of each gene included in Table 2 was analyzed using the TaqMan chemistry (Thermo Fisher Scientific, Waltham, Massachusetts, USA). Each RT-qPCR sample contained 4 µL cDNA, 10 µL TaqMan Gene Expression Master Mix, 1 µL TaqMan Gene Expression Assay (Table 2) and 5 µL RNase-free water. For each RNA isolation (one for each cell culture well), duplicate (or triplicate) amplifications of the corresponding cDNA were performed. In addition, one NRT sample from each RNA isolate was included for each gene to check for genomic DNA

Table 2: Candidate reference and target genes evaluated in this thesis

Gene symbol	Accession number [a]	Product number TaqMan gene expression assay
GAPDH	NM-001256799.1	Hs02758991_g1
18S rRNA	NR-003286.2	Hs99999901_s1
B2M	NM-004048.2	Hs00984230_m1
ACTB	NM-001101.3	Hs01060665_g1
NF-κB1	NM-001165412.1	Hs00765730_m1
PTGS2	NM-000963.2	Hs00153133_m1
NFE2L2	NM-001145412.2	Hs00975961_g1

[a]Source: NCBI reference sequence database.

contaminations. The probe was of type FAM/MGB. For each gene (target and reference gene) non-template controls were included. No amplification signals were obtained when RNase-free water was added instead of cDNA indicating that the reaction mixture was not contaminated by DNA. The RT-qPCR was performed on an ABI PRISM 7900HT instrument (Applied Biosystems, Foster City, California, USA) with the following temperature setting: (i) 95 °C for 10 minutes, (ii) 40 cycles 95 °C for 15 seconds and 60 °C for 1 minute. The amplification plot and SDS-software were used to ontain the C_T values.

2.5 Data analysis

2.5.1 PCR amplification efficiency

Theory Constant PCR efficiencies in target and reference genes are required in RT-qPCR assays. Equal PCR efficiencies are particularly important when differences in target gene concentrations are based on normalization to a reference gene as it is the case for the $\Delta\Delta C_T$ method (Bustin et al., 2009). Differences in efficiencies between target and reference genes can result in over or under-estimations of expression ratios. The PCR efficiency should be as close to 100% as possible. A 100% efficiency means that the cDNA target is doubled in every PCR cycle. The PCR efficiency can be affected by not properly designed primers or probes as well as suboptimal PCR conditions.

The PCR efficiency can be determined by

$$N = N_0 \cdot E^{n_c}, \tag{2}$$

where N is the number of amplified molecules, N_0 is the initial number of molecules, E is the efficiency and n_c the number of PCR cycles. Equation (2) can be rearranged to

$$log(N) = log(N_0) + n_c \cdot log(E), \tag{3}$$

$$n_c = \frac{log(N) - log(N_0)}{log(E)}, \tag{4}$$

and

$$n_c = -\frac{1}{log(E)} \cdot log(N_0) + \frac{log(N)}{log(E)}. \tag{5}$$

Standard curves can be described by equation (5). The slope of the standard curve is

$$slope = -\frac{1}{log(E)} \tag{6}$$

and can be used to calculate the efficiency

$$E = 10^{-slope^{-1}}. \tag{7}$$

The efficiency of a PCR is optimal if the cDNA target is doubled every PCR cycle. The slope of the standard curve is -3.32 in the optimal case. The slope is estimated using linear regression analysis of the C_T values explained by the cDNA dilution.

The efficiency is given as a percentage value

$$\%E = (E - 1) \cdot 100. \tag{8}$$

An efficiency ($\%E$) of 90% to 110% can be considered as good (Garson et al., 2009).

Application The efficiency of the PCR amplification of the four candidate reference genes *GAPDH*, *18S rRNA*, *B2M* and *ACTB* was verified. To do so, a pooled cDNA sample was prepared by taking equal amounts of all treated and untreated cDNA samples from one cell culture experiment. The PCR efficiency was determined by three-point standard curves of a 10-fold dilution series (1:1 - 1:100). The threshold cycles obtained in the RT-qPCR were plotted against the concentration of the samples in logarithmic scale. Given an efficiency of 100%, the C_T will increase linearly with the decreasing quantity of the target. The efficiency was determined by generating a standard curve and estimating the slope. Each dilution was amplified in duplicates and an average was taken.

If not mentioned otherwise, all statistical analysis were carried out in the R software for statistical programing (R Core Team, 2015).

2.5.2 Stability of reference genes

Theory The basis for the normalization of target genes is to identify reference genes whose mRNA expression level does not change significantly between different treatments. As advised by Kozera and Rapacz (2013), three different validation methods were used to determine the stability of the reference genes: (i) Analysis of variance (ANOVA) and Tukey honest significant difference (HSD) test, (ii) BestKeeper software, and (iii) NormFinder software.

By using an ANOVA it can be determined whether mean expression levels given treatments in a gene differ significantly from each other or not. For further comparison (multiple comparisons) among the groups of treatment means, the Tukey HSD test can be applied. For both tests the following assumptions need to be satisfied: (i) the tested observations are independent both within and among the groups, (ii) the data are normally distributed, and (iii) the variance within the compared groups is equal.

The BestKeeper software (Pfaffl et al., 2004) is an Excel-based tool, that can be used to determine the stability of reference genes. From the C_T values, the geometric and arithmetic mean, the standard deviation and the coefficient of variation are derived. The BestKeeper software ranks the reference genes by their standard deviation (Maroufi et al., 2010). The most stable reference genes are the ones with lowest standard deviation. Only reference genes with a standard deviation below 1 should be included in further analysis (Pfaffl et al., 2004). Repeated pairwise correlation analysis of the reference genes are made to estimate the inter-gene relations resulting in coefficient of correlation values (r). The reference genes with the strongest correlations are combined into the BestKeeper index. The BestKeeper index is the geometric mean of those genes. To evaluate the stability of the individual reference genes pair-wise, the correlations between the reference genes and the BestKeeper index (BKI) are calculated.

NormFinder is a free Excel-addin (Andersen et al., 2004) that can be used to identify the stability of reference genes. The tested reference genes are ranked according to their stability. Furthermore, intra- and intergroup variations can be studied (Kozera and Rapacz, 2013). According to Andersen et al. (2004), expression variations in the reference genes have to be considered. All reference genes will show some variations due to different groups in a sample, e.g. normal and tumor tissue, or other biological or experimental factors. The idea behind the NormFinder algorithm is to select reference genes based on a model-based approach. By using this approach, it is possible to estimate the overall expression variation of reference genes and also variations between subgroups of a sample set. The stability of reference genes is indicated by a stability value.

Application For validating the stability of the reference genes in the experimental conditions, the ANOVA and Tukey HSD test was used. The assumptions needed to be fulfilled for using these tests. To check if the data are normally distributed, the Shapiro-Wilk normality test was used and to evaluate the assumption of equal variances, the Levene test was applied. The Tukey HSD test was only applied if the ANOVA revealed that the treatment means in a gene differ significantly from each other.

To determine the stability of the reference genes by using the BestKeeper software, for each experiment the average C_T values from all reactions were used to calculate the standard deviation. Reference genes with standard deviations below 1 were used in further analysis. For reference genes that fulfill this criteria, the pairwise correlation between the individual reference gene C_T value and the BestKeeper index was calculated using the BestKeeper software.

For determining the stability of the potential reference genes by using NormFinder software, the input data have to be on a linear scale and raw C_T values cannot be used directly. Since the amplification efficiency of the reference genes was approximately 100%, the C_T values were linearized by 2^{-C_T} and the data were entered into NormFinder (Andersen et al., 2004).

2.5.3 Normalization process

Theory Single or combined reference genes that are expressed at a constant level given treatments are used to normalize the gene expression of the target gene. The reference genes used for normalization depend on the results obtained in the previous step in which the stability of the reference genes is tested. The arithmetic mean of the most stable reference genes can be used to combine reference genes.

Application Many experiments were carried out before the normalization analysis was finalized. In these experiments, only *18S rRNA* and *GAPDH* were used as reference genes. In order to analyze the influence of using single, potentially unstable reference genes, the normalization analysis were carried out using the following set-up: (i) arithmetic mean of *18S rRNA*, *B2M*, and *ACTB*, (ii) arithmetic mean of *GAPDH*, *18S rRNA*, *B2M*, and *ACTB*, (iii) only *GAPDH*, and (iv) only *18S rRNA*.

2.5.4 $2^{-\Delta\Delta C_T}$ method

Theory Data from RT-qPCR experiments can be analyzed using the $2^{-\Delta\Delta C_T}$ method which is also called Livak method (Livak and Schmittgen, 2001). This method assumes that the amplification efficiencies of target and reference are near 100%. Table 3 shows the required C_T values when

Table 3: Required information for relative quantification using reference genes as normalizers.

Sample	Gene	
	Target gene (tar)	Reference gene (ref)
Control (con)	$C_{T(tar,con)}$	$C_{T(ref,con)}$
Treatment (treat)	$C_{T(tar,treat)}$	$C_{T(ref,treat)}$

Adapted from Bio-Rad Laboratories (2006)

relative quantification is normalized by reference genes and the $2^{-\Delta\Delta C_T}$ method is used. In the following the $2^{-\Delta\Delta C_T}$ method will be described for the most simple example – one target and one reference gene, one test and one calibrator sample.

The $2^{-\Delta\Delta C_T}$ method consists of three steps. In the first step the C_T value of the target gene is normalized to the C_T value of the reference gene

$$\Delta C_{Tcon} = C_{T(tar,con)} - C_{T(ref,con)} \tag{9}$$

$$\Delta C_{Ttreat} = C_{T(tar,treat)} - C_{T(ref,treat)}. \tag{10}$$

In the second step ΔC_{Ttreat} is normalized given ΔC_{Tcon}

$$\Delta\Delta C_T = \Delta C_{Ttreat} - \Delta C_{Tcon}. \tag{11}$$

In the third and last step, the fold change is calculated as the expression difference between the two genes in the two samples

$$Fold\ change = 2^{-\Delta\Delta C_T}. \tag{12}$$

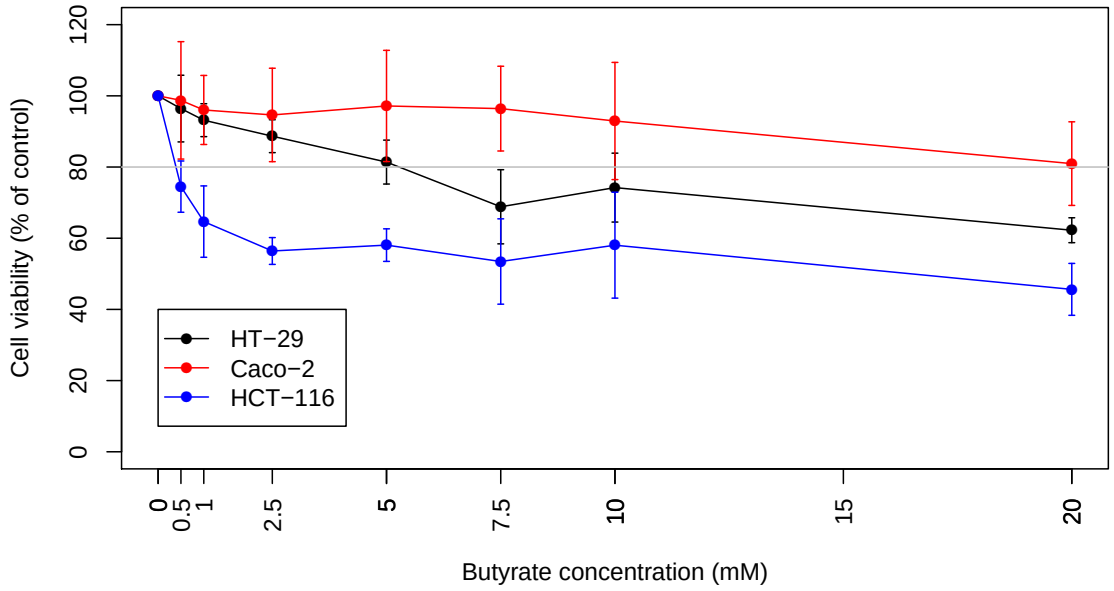


Figure 9: The inhibitory effect of different butyrate concentrations on cell proliferation of HT-29, Caco-2 and HCT-116 cells using the MTT assay. The graph shows the mean and the standard deviation (n=6) for three independent cell experiments with two parallels. The horizontal gray line at 80% cell viability indicates the highest acceptable inhibitory effect of butyrate on the cell lines.

3 Results

3.1 MTT assay

The MTT assay was used to test the cytotoxic effect of different butyrate concentrations on colon carcinoma cells of the cell lines HT-29, Caco-2 and HCT-116. The cells were treated with butyrate at various concentrations (0.5, 1.0, 2.5, 5.0, 7.5, 10.0 and 20.0 mM). The cell viability was determined after 24 hours with treatment (Figure 9). The butyrate concentration was considered to be too toxic, if the cell proliferation was inhibited by more than 80%. Even though all three cell lines responded differently to the butyrate treatment, the cell viability decreased in a dose-dependent manner for all. Treatment with 20 mM butyrate inhibited the growth of HCT-116 cells by approximately 55% while the same butyrate concentration reduced the cell viability of HT-29 and Caco-2 cells by 40% and 20%, respectively. To allow comparability, a butyrate concentration of 2.5 mM was chosen for all following experiments. The treatment time for HCT-116 cells in later experiments was less than 24 hours and therefore 2.5 mM butyrate was assumed as suitable.

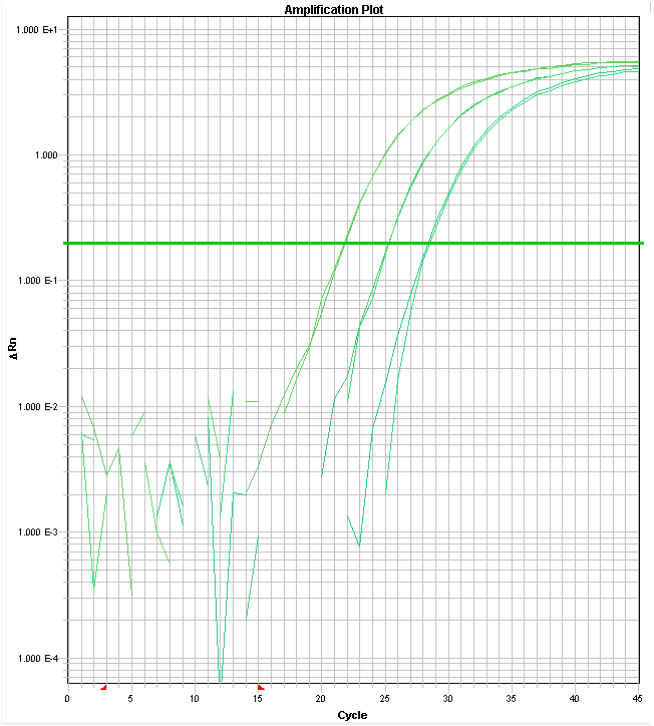


Figure 10: Amplification plot for *ACTB* for a 10-fold dilution series visualized in the software SDS version 2.4.

3.2 RT-qPCR

3.2.1 Experimental treatment for 24 hours on HT-29 cells

PCR amplification efficiency One assumption in RT-qPCR is that the PCR efficiency is equal for target and reference genes. The PCR efficiency can be affected by not properly designed primers or probes as well as suboptimal PCR conditions. A pooled cDNA sample from one HT-29 cell experiment with 24 hours of incubation was therefore used for a crude evaluation of PCR efficiencies of the four candidate reference genes by constructing a three-point standard curve with 10-fold dilutions. Figure 10 shows the amplification plots of the dilution series of *ACTB*.

The obtained threshold cycles of the *ACTB* dilution series are plotted against the dilution of the sample (Figure 11).

Properties of the estimated standard curves for the analyzed reference genes are shown in Table 4. All reference genes, except *18S rRNA*, were within the acceptable %E range of 100% ± 10%. *18S rRNA* was with 111% just slightly outside the acceptable range.

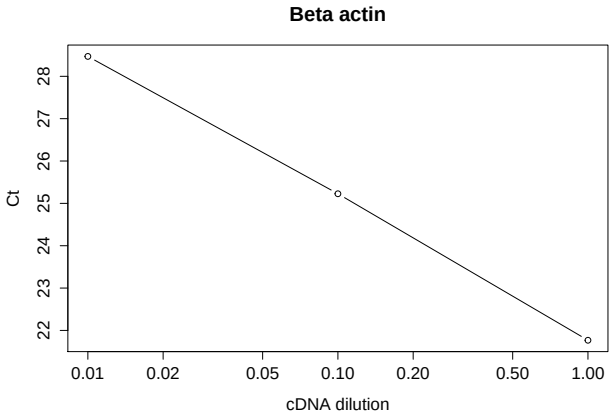


Figure 11: Standard curve for *ACTB* with three data points covering a 10-fold dilution series. The value for the slope is -3.351 and the coefficient of determination 0.99.

Table 4: Standard curve and efficiency for reference genes.

Gene	Slope	Coefficient of determination	Efficiency (%)
GAPDH	-3.435	0.994	95.5
18S rRNA	-3.084	0.998	111.0
B2M	-3.169	0.999	107.0
ACTB	-3.351	0.999	98.8

Stability of reference genes in the HT-29 cell line The stability of the four reference genes *GAPDH*, *18S rRNA*, *B2M*, and *ACTB* was evaluated in HT-29 cells treated for 24 hours with either 2.5 mM butyrate, $1\,\mathrm{mg\,mL^{-1}}$ dispersible wellmune, or untreated medium (see Table 1 for technical or biological replicates). The expression level of the reference genes was measured using RT-qPCR.

The variations among the C_T values for each treatment are shown in Figure 12. This figure allows a visual assessment of the potential reference genes within the experimental treatments. *B2M* and *ACTB* clearly show more variations in their expression levels across the different treatments than *18S rRNA* and *GAPDH*. In order to support the visual inspection (Figure 12) by statistical inference, an ANOVA followed by a Tukey HSD test was applied. Shapiro-Wilk's test and the Levene's test were used to evaluate the assumptions of normally distributed data and equal variances, which are essential for a correct application of ANOVA. The ANOVA showed that there was no significant difference in gene expression between butyrate, dispersible wellmune and no treatment at a 0.05 level of significance. Therefore the Tukey test was not needed. The Shapiro-Wilk's and Levene's test suggested that the assumptions of the ANOVA were not violated.

The BestKeeper software determines the most stable reference genes by calculating the standard deviation of input C_T values and the pair-wise correlation analysis between reference genes and the

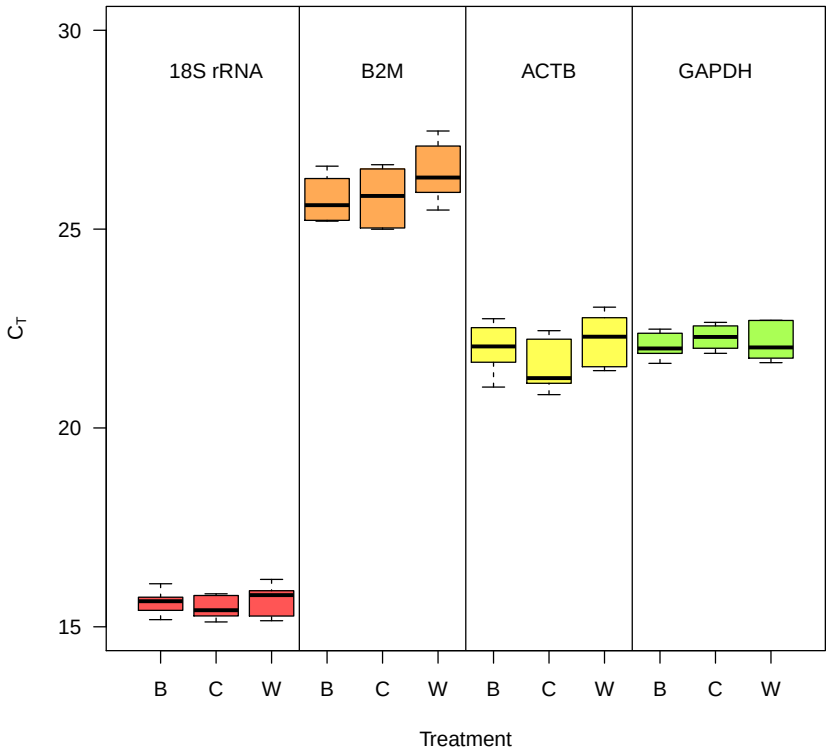


Figure 12: Boxplot of C_T values from RT-qPCR analysis of the four reference genes *18S rRNA*, *B2M*, *ACTB*, and *GAPDH* in HT-29 cells after 24 hours with butyrate (denoted B), dispersible wellmune (denoted W), and no treatment (denoted C). The horizontal lines within the boxes represent the median expression levels of the candidate reference genes while the boxes represent the 25% and 75% percentiles. The whiskers represent the range of the expression levels. Each box represents six data points resulting from three independent experiments.

Table 5: Descriptive statistics of the C_T for the four reference genes using BestKeeper software (n=17, resulting from three independent experiments).

	GAPDH	*18S rRNA*	*B2M*	*ACTB*
GM	22.35	15.74	26.01	21.90
SD	0.43	0.29	0.59	0.61
CV	1.92	1.83	2.27	2.78

Abbreviations: n = number of samples, GM = geometric mean of C_T, SD = standard deviation of C_T, CV (%) = coefficient of variation (SD expressed as percentage of GM).

Table 6: Stability of potential reference genes in HT-29 cells using BestKeeper (n=17) and NormFinder (n≥5).

Gene name	Stability value (BestKeeper)	Ranking (BestKeeper)	Stability value (NormFinder)	Ranking (NormFinder)
GAPDH	-0.32	4	0.24	4
18S rRNA	0.82	2	0.09	1
B2M	0.94	1	0.18	3
ACTB	0.62	3	0.16	2

A ranking of 1 indicates the most stable reference gene.

BestKeeper index. The lowest variation was observed for *18S rRNA* while *ACTB* had the highest variation, with standard deviations of 0.29 and 0.61, respectively (Table 5).

All tested reference genes had standard deviations below 1. Therefore, all reference genes were used for further analysis. The highest correlations were obtained for *B2M* and *18S rRNA*. *GAPDH* was ranked as the least stable reference gene (Table 6).

The NormFinder software selects the most stable reference genes using a model-based approach. The results were partly in accordance with the results obtained with BestKeeper but showed also differences. For instance, *B2M* which was ranked as the most stable reference gene in the BestKeeper software, was only the third-most stable reference gene in the NormFinder software. The most stable reference gene with the lowest stability value was *18S rRNA*. *GAPDH* was identified as the least stable gene by NormFinder which is in accordance with the results in the BestKeeper software (Table 6). Additionally, *GAPDH* had the highest inter- and intragroup variations.

According to the results of BestKeeper and NormFinder, *GAPDH* – one of the most commonly used reference genes – should be avoided for the normalization in this study. The BestKeeper software requires manually elimination of less suitable reference genes to avoid stability variations that are biased due to high intergroup variations. The BestKeeper analysis was therefore repeated without *GAPDH*. The correlation between the remaining genes increased clearly ($0.85 < r < 0.97$). It can be concluded that *GAPDH* should not be used as reference gene.

Fold changes after experimental treatment for 24 hours on HT-29 cells In order to evaluate the practical significance of the results above, normalization of target gene expression in

Table 7: Descriptive statistics of fold changes given different treatments. Each statistic is based on six observations consisting of three experiments and two biological parallels.

	Gene	*PTGS2*		*NF-κB1*		*NFE2L2*	
	Treatment	B	W	B	W	B	W
3 reference genes	Mean	0.32	0.48	0.24	0.53	0.92	1.91
	SD	0.08	0.19	0.03	0.09	0.19	0.44
	p-value	<0.001	0.007	<0.001	0.001	0.24^{ns}	0.013
4 reference genes	Mean	0.31	0.43	0.23	0.47	0.87	1.69
	SD	0.08	0.18	0.03	0.09	0.19	0.44
	p-value	<0.001	0.004	<0.001	0.001	0.133^{ns}	0.025
GAPDH	mean	0.27	0.38	0.18	0.30	0.66	1.05
	SD	0.07	0.23	0.03	0.08	0.18	0.38
	p-value	<0.001	0.001	<0.001	<0.001	0.03	0.393^{ns}
18S rRNA	Mean	0.33	0.39	0.25	0.45	0.94	1.63
	SD	0.08	0.15	0.02	0.12	0.19	0.49
	p-value	<0.001	0.002	<0.001	0.002	0.298^{ns}	0.041

p-values indicate the significance level of one-sided t-tests for the difference between the mean and 1.

Abbreviations: SD = Standard deviation; ns = not significant at the 95% confidence level; 3 reference genes = arithmetic mean of *18S rRNA*, *ACTB*, *B2M*; 4 reference genes = arithmetic mean of *GAPDH*, *18S rRNA*, *ACTB*, *B2M*; Treatment = cells treated with butyrate (denoted B) or dispersible wellmune (denoted W).

HT-29 cells treated with butyrate or dispersible wellmune for 24 hours was performed in different ways. First, the arithmetic mean of C_T values of *18S rRNA*, *B2M*, and *ACTB* was used for normalization. The results of this normalization are shown in (Figure 13A and Table 7). A significant down-regulation of the gene expression of *NF-κB1* and *PTGS2* in response to butyrate and dispersible wellmune can be seen there. *NFE2L2* gene expression on the other hand was up-regulated by dispersible wellmune, but unaffected by butyrate.

Using all four reference genes for normalization, resulted in essentially the same as using three reference genes (Figure 13B and Table 7).

The same was the case if *18S rRNA* was used as single reference gene for normalization (Figure 13D and Table 7).

By using *GAPDH* as a single reference gene for normalization the same results as for normalization with the three most stable or all four reference genes were obtained for *NF-κB1* and *PTGS2*. However in contrast to this, *NFE2L2* appeared to be significantly down-regulated in the butyrate treated samples and not significantly up-regulated in the dispersible wellmune treated samples (Figure 13C and Table 7).

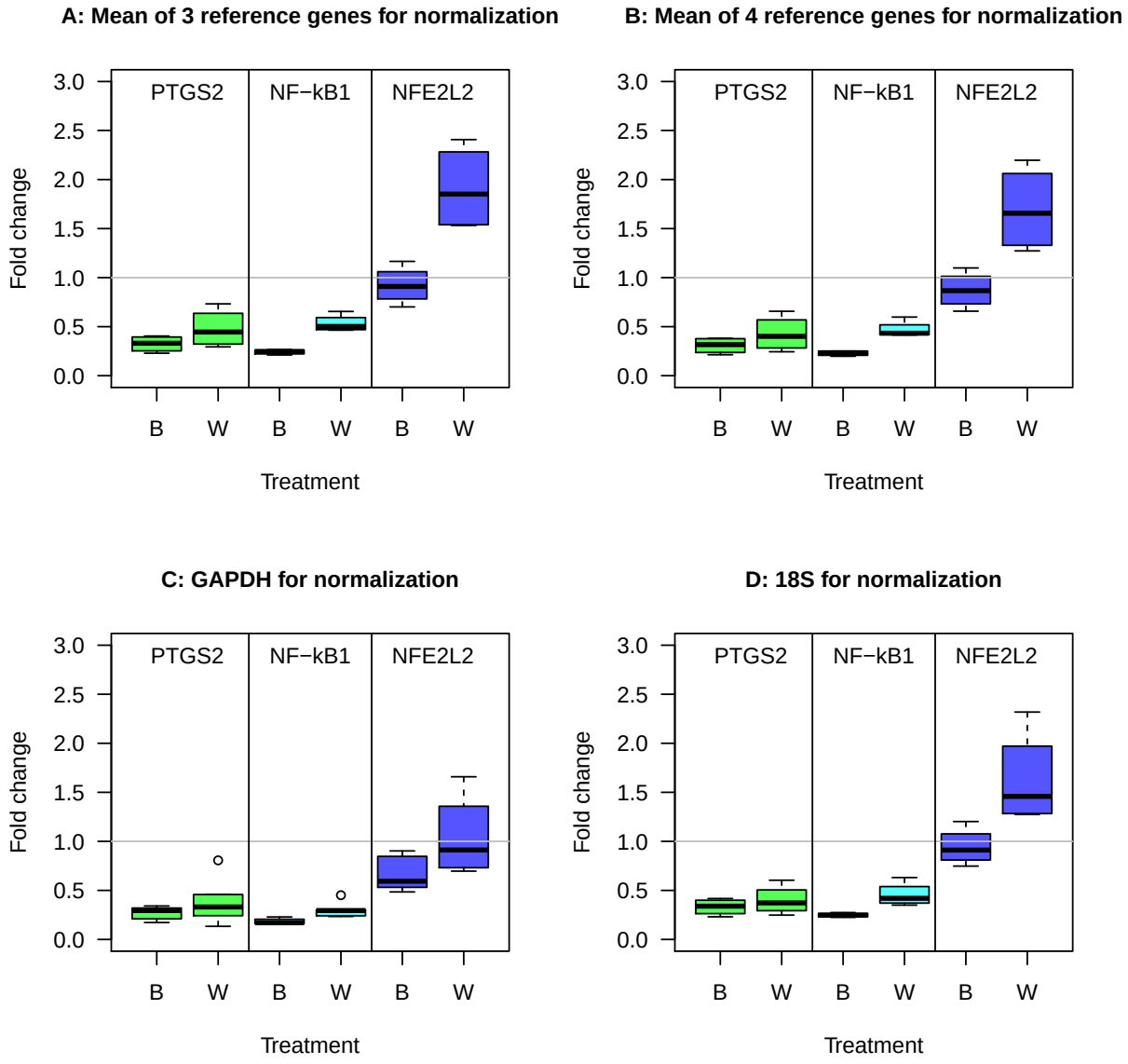


Figure 13: Boxplots of fold changes resulting from RT-qPCR analysis of *PTGS2*, *NF-κB1*, and *NFE2L2* in HT-29 cells after 24 hours treatment with butyrate (denoted B) or dispersible wellmune (denoted W). Each box represents six data points resulting from three independent experiments. The horizontal lines within the boxes represent the median expression levels of the candidate reference genes while the boxes represent the 25% and 75% percentiles. The whiskers represent the range of the expression levels. **A**: The arithmetic mean of *18S rRNA*, *B2M*, and *ACTB* was used for normalization. **B**: The arithmetic mean of *GAPDH*, *18S rRNA*, *B2M*, and *ACTB* was used for normalization. **C**: *GAPDH* was used for normalization. The circles indicate potential outliers which are more than 1.5 times the interquartile range above the box. **D**: *18S rRNA* was used for normalization.

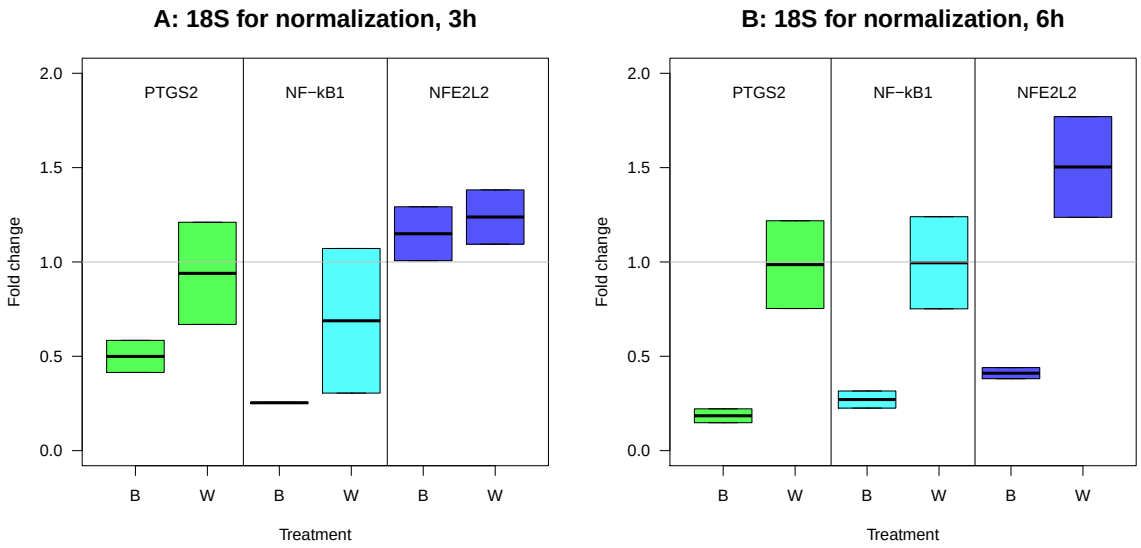


Figure 14: Boxplots of fold changes resulting from RT-qPCR analysis of *PTGS2*, *NF-κB1*, and *NFE2L2* in HT-29 cells after treatment with butyrate (denoted B) or dispersible wellmune (denoted W). Each box is based on two observations resultnig from one experiment and two biological parallels. The horizontal lines within the boxes represent median expression levels while the boxes represent the 25% and 75% percentiles. **A**: *18S rRNA* was used as reference gene. Treatment time was three hours. **B**: *18S rRNA* was used as reference gene. Treatment time was six hours.

3.2.2 Experimental treatment for three and six hours on HT-29 cells

Fold changes after experimental treatment for three or six hours on HT-29 cells In a pre-experiment on different incubation times, the gene expression of *PTGS2*, *NF-κB1*, and *NFE2L2* was studied by using only *18S rRNA* for normalization. HT-29 cells were treated for three hours or six hours with butyrate or dispersible wellmune. The cell experiment was conducted only once (see Table 1). Due to the small number of observations (n=2) no statistical analysis was performed, but the results are displayed graphically in Figure 14. Relative changes in the gene expression of the target genes after three hours with treatment are shown in Figure 14A. *PTGS2* and *NF-κB1* gene expression were down-regulation after three hours incubation with butyrate while no distinct changes were observable for the dispersible wellmune treatment. The range in the fold change of both genes after the dispersible wellmune treatment was considerably larger than for the butyrate treatment. *NFE2L2* displayed a slight tendency for up-regulation after both treatments.

In Figure 14B, the relative gene expression of the three genes of interest is shown after six hours with butyrate or dispersible wellmune treatment. The gene expression of these genes was inhibited by the butyrate treatment. As for the dispersible wellmune treatment for three hours, there is no change in the gene expression level in *PTGS2* and *NF-κB1* after six hours. The tendency of an increased *NFEL2L* gene expression observed after three hours treatment with dispersible wellmune

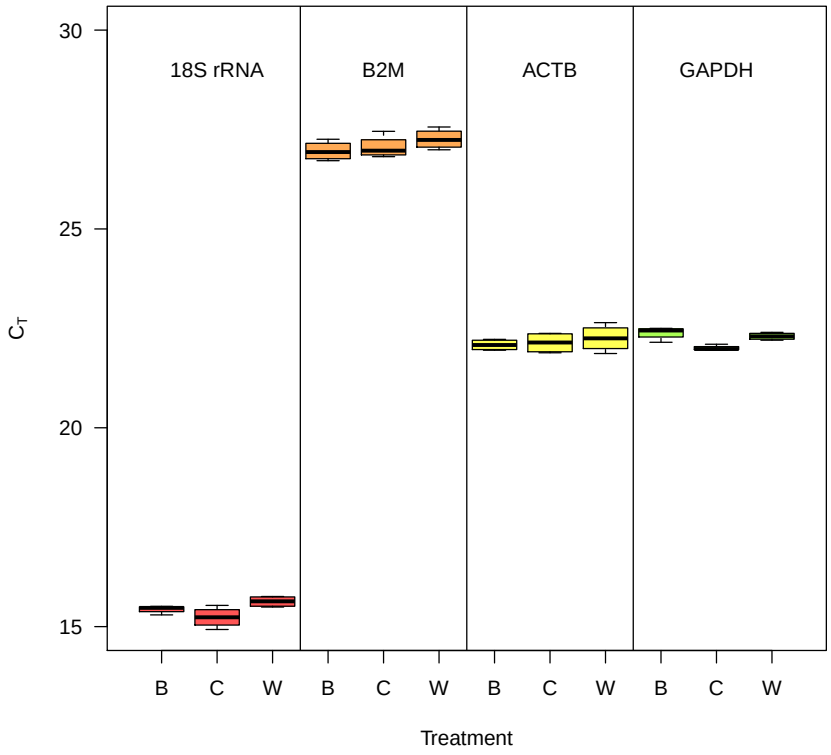


Figure 15: Boxplot of C_T values from RT-qPCR analysis of the four reference genes *18S rRNA*, *B2M*, *ACTB*, and *GAPDH* in HCT-116 cells after six hours with butyrate (denoted B), dispersible wellmune (denoted W), and no treatment (denoted C). The horizontal lines within the boxes represent the median expression levels of the reference genes while the boxes represent the 25% and 75% percentiles. The whiskers represent the range of the expression levels. Each box represents four data points resulting from two independent experiments.

is strengthened after six hours. Again, the range in the fold change in the target genes after the dispersible wellmune treatment was considerably larger than after the butyrate treatment.

3.2.3 Experimental treatment for six hours on HCT-116 cells

Stability of reference genes in HCT-116 cell line After treating HCT-116 cells for six hours with either 2.5 mM butyrate or $1\,\mathrm{mg\,mL^{-1}}$ dispersible wellmune, the stability of the four reference genes *GAPDH*, *18S rRNA*, *B2M*, and *ACTB* was evaluated. The expression level of the reference genes was measured using RT-qPCR and their stability was tested by three different approaches.

Figure 15 shows the variations among the C_T values for each treatment. This figure allows a visual assessment of the potential reference genes given the experimental treatments. The ANOVA revealed no significant difference between treatments in the genes *B2M* and *ACTB* while significant differences occurred in *GAPDH* and *18S rRNA* (Table 8). The Tukey HSD test clarified that

in *GAPDH*, the gene expression level between the control and the butyrate and dispersible wellmune treatment were significantly different. There was no significant difference in gene expression between dispersible wellmune and butyrate. In *18S rRNA*, the dispersible wellmune treatment differed significantly from the control. There were no significant differences between the other treatments (Table 8).

Table 8: ANOVA and Tukey HSD tests measurements of significant group mean differences of reference genes in HCT-116 cells.

Gene name	p-value ANOVA	Treatment comparision	p-value Tukey HSD test
GAPDH	0.009^s	C-B	0.008^s
		W-B	0.596
		W-C	0.031^s
18S rRNA	0.034^s	C-B	0.282
		W-B	0.317
		W-C	0.027^s
B2M	0.337	C-B	0.872
		W-B	0.286
		W-C	0.527
ACTB	0.654	C-B	0.955
		W-B	0.641
		W-C	0.806

p-values indicate the significance level of ANOVA or Tukey HSD test for the difference between the various treatments in a gene. Abbreviations: s = significant difference between treatments in the specific gene on a 95% significance level, C = control, W = dispersible wellmune, B = butyrate.

The BestKeeper software showed the lowest variation for *GAPDH* while *ACTB* had the highest variation, with standard deviations of 0.15 and 0.22, respectively (Table 9). Since the standard deviation for all reference genes was below 1, all reference genes were used for further analysis. The BestKeeper software revealed that the highest correlations were obtained for *18S rRNA* and *B2M* while *GAPDH* was ranked as the least stable reference gene (Table 10). BestKeeper suggests that *GAPDH* should be avoided as reference gene for normalization. When omitting *GAPDH* as the least stable reference gene from the BestKeeper analysis, the correlation between the remaining genes increased slightly ($0.65 < r < 0.88$).

The results of the NormFinder software were partly in accordance with the results obtained using BestKeeper. *GAPDH* was identified as the least stable reference gene by both softwares. Its stability value was however only slightly worse than for the other genes. Additionally, *GAPDH* had the highest inter- and intragroup variation. *ACTB* was ranked as the most stable reference gene in the NormFinder software while it was only the third most stable reference gene when the BestKeeper software was used.

As for the HT-29 cells, it can be concluded that *GAPDH* is the least stable reference gene.

Table 9: Descriptive statistics of C_T values in HCT-116 cells for four candidate reference genes using BestKeeper software (n=12).

	GAPDH	*18S rRNA*	*B2M*	*ACTB*
GM	22.25	15.41	27.09	22.16
SD	0.15	0.16	0.20	0.22
CV	0.67	1.04	0.91	0.80

Abbreviations: n = number of samples, GM = geometric mean of C_T, SD = standard deviation of C_T, CV = coefficient of variation (SD expressed as percentage of the geometric mean).

Table 10: Stability of potential reference genes in HCT-116 cells using BestKeeper (n=12) and NormFinder (n=4).

Gene name	Stability value (BestKeeper)	Ranking (BestKeeper)	Stability value (NormFinder)	Ranking (NormFinder)
GAPDH	0.19	4	0.09	4
18S rRNA	0.78	1	0.07	2
B2M	0.74	2	0.08	3
ACTB	0.59	3	0.06	1

A ranking of 1 indicates the most stable reference gene.

Fold changes after experimental treatment for six hours on HCT-116 cells The amplification of *PTGS2* occurred later than expected. The C_T values were between 35 and 37 (Table 11). For TaqMan assays the cut-off is 35 cycles. Everything above this number of cycles is considered to be unreliable detections of gene expressions. Since the C_T values of *PTGS2* were above the acceptable cut-off level, the data were not used for fold change calculations.

When RT-qPCR was performed on cDNA from HCT-116 cells treated with butyrate for six hours and the arithmetic mean of *18S rRNA*, *B2M*, and *ACTB* was used for normalization, the treatment led to a significant down-regulation of *NF-κB1* and *NFE2L2*. The treatment of HCT-116 cells with dispersible wellmune led to no significant changes inmregulation of the *NF-κB1* gene, while *NFE2L2* was significantly up-regulated (Figure 16A and Table 12).

The BestKeeper software ranked *GAPDH* as unstable reference gene. To determine the impact of using *GAPDH* anyways, the arithmetic mean of all reference genes was used for normalization. As illustrated in Figure 16 and Table 12, the use of three (without *GAPDH*), four (with *GAPDH*) or only one (*GAPDH*) reference gene for normalization had little impact on the resulting fold change expressions of the target genes. All three normalization alternatives revealed a significant reduction of *NF-κB1* and *NFE2L2* gene expression by butyrate treatment and a significant increase

Table 11: Mean and standard deviations of C_T values in HCT-116 cells after six hours with butyrate, dispersible wellmune, and no treatment (control) (n=4).

Treatment	Mean	Standard deviation
Control	36.84	0.21
Butyrate	34.82	0.13
Dispersible wellmune	35.07	0.39

Table 12: Descriptive statistics of fold changes given different treatments in HCT-116 cells. Each statistic is based on four observations consisiting of two experiments and two parallels.

	Gene	*NF-κB1*		*NFE2L2*	
	Treatment	B	W	B	W
3 reference genes	Mean	0.51	0.93	0.45	1.64
	SD	0.05	0.19	0.04	0.18
	p-value	<0.001	0.253^{ns}	<0.001	0.003
4 reference genes	Mean	0.56	0.97	0.51	1.72
	SD	0.07	0.14	0.08	0.06
	p-value	<0.001	0.364^{ns}	0.001	<0.001
GAPDH	mean	0.61	0.91	0.55	1.59
	SD	0.01	0.18	0.01	0.99
	p-value	0.005	0.179^{ns}	0.003	0.003

p-values indicate the significance level of one-sided t-tests for the difference between the mean and 1.

Abbreviations: SD = Standard deviation; ns = not significant at the 95% confidence level; 3 reference genes = arithmetic mean of *18S rRNA*, *ACTB*, *B2M*; 4 reference genes = arithmetic mean of *GAPDH*, *18s rRNA*, *ACTB*, *B2M*; Treatment = cells treated with butyrate (denoted B) or dispersible wellmune (denoted W).

in *NFE2L2* gene expression by dispersible wellmune treatment. No significant effect of dispersible wellmune treatment in *NF-κB1* gene expression could be observed.

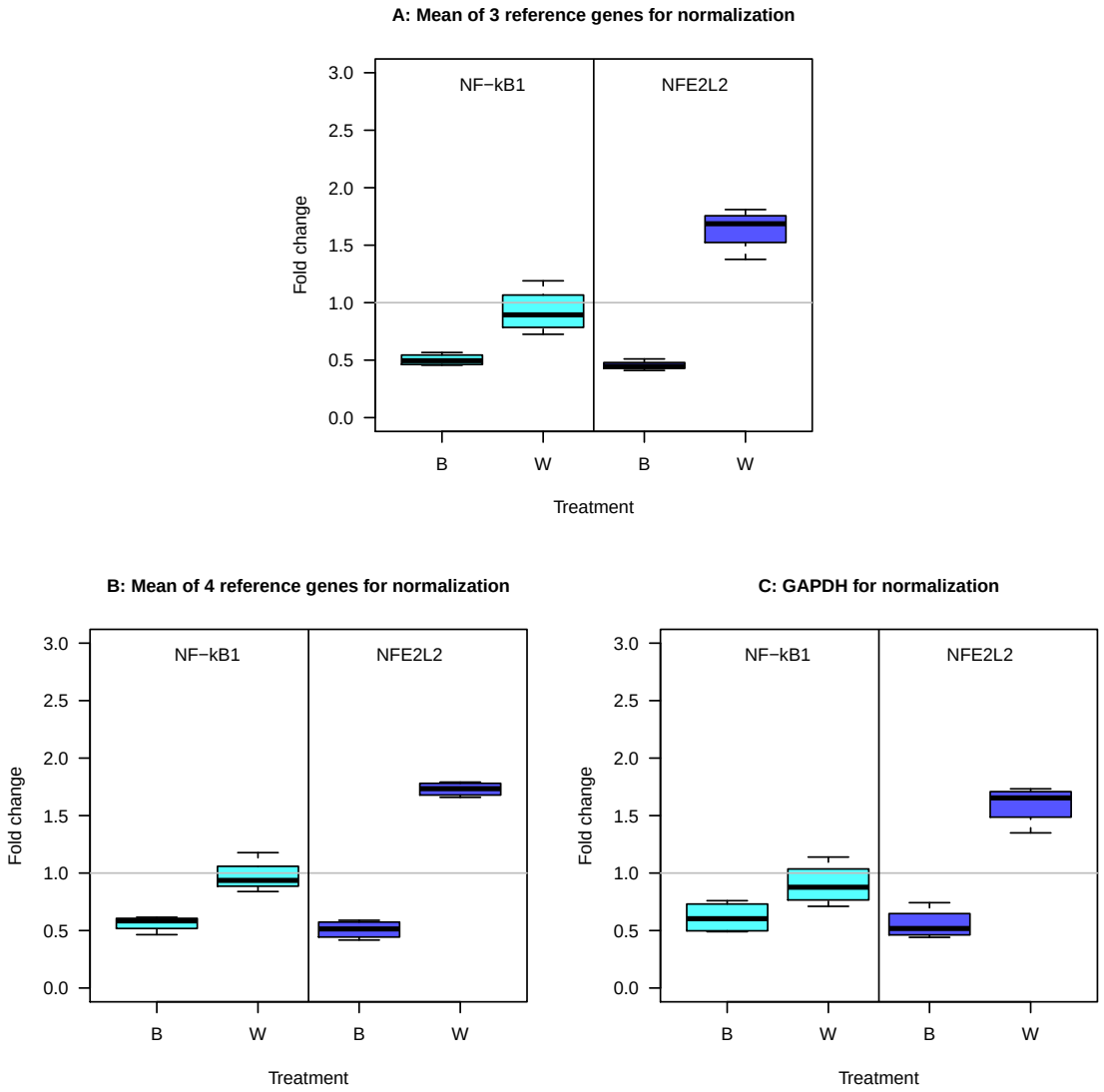


Figure 16: Boxplots of fold changes resulting from RT-qPCR analysis of *NF-κB1* and *NFE2L2* in HCT-116 cells after six hours treatment with butyrate (denoted B) or dispersible wellmune (denoted W). Each box represents four data points resulting from two independent experiments. The horizontal lines within the boxes represent the median expression levels of the reference genes while the boxes represent the 25% and 75% percentiles. The whiskers represent the range of the expression levels. **A**: The arithmetic mean of *18S rRNA*, *B2M*, and *ACTB* was used for normalization. **B**: The arithmetic mean of *GAPDH*, *18S rRNA*, *B2M*, and *ACTB* was used for normalization. **C**: *GAPDH* was used for normalization.

4 Discussion

4.1 MTT assay

To study the effect of butyrate as one of the important dietary fiber fermentation metabolites on key aspects of gene expression in colon cancer cell lines, a butyrate concentration was needed which was high enough to cause alterations in gene expression and low enough to avoid apoptosis. The MTT assay was used to find a concentration that fulfilled these constraints by analyzing changes in the cell viability due to different butyrate concentrations. The butyrate concentration was assumed to be too toxic if the cell viability dropped below 80% after 24 hours with treatments in HT-29, HCT-116, and Caco-2 cells. For HT-29 cells, concentrations up to 2.5 mM butyrate fulfilled the 80% requirement. These results are in accordance with the study of Siavoshian et al. (2000), who demonstrated that HT-29 cells, treated with butyrate for three days started an inhibition in cell growth at 1 mM. A complete inhibition was observed at a concentration of 8 mM butyrate.

In this thesis, Caco-2 cells were not found to be responsive to butyrate treatment after 24 hours and were therefore not considered in further analysis. Blok (2002, p.80) treated Caco-2 cells for two to 19 days with butyrate and observed that 2 mM butyrate had no effect on cell proliferation. A strong cell growth inhibiting effect was seen with 10 mM butyrate in the same time-period. This suggests that Caco-2's response to butyrate treatment is slower which could be considered in future studies.

Butyrate reduced HCT-116 cell proliferation at very low levels in this thesis. Already the lowest butyrate concentration tested, resulted in a cell proliferation below the 80% requirement. The study of Fung et al. (2011) supports this observation. In their study, a 2.5 mM butyrate treatment for 24 hours induced cell apoptosis and inhibited cell proliferation. In the present study, HCT-116 cells reacted more sensitive on a butyrate treatment than HT-29 and Caco-2 cells. According to ATCC, the provider of the cell lines, HT-29 and Caco-2 cells have mutations in the *p53* gene, while HCT-116 has not. This could be one of the reasons for the observed different responsiveness of the cell lines to butyrate, because differences in *p53* gene mutations have previously been shown to be important for specific cell line characteristics. Yao et al. (2005) have, for example, tested the adaption of tumor cells to hypoxia (lower oxygen concentration in the arterial blood than normal) by recreating *in vitro* situations of chronic exposure to low-oxygen levels. They found that HT-29 cells became more resistant to hypoxia while HCT-116 cells became more sensitive to hypoxia. The difference in the *p53* gene between the cell lines was hypothesized to account for the contrary directions of sensitivity to hypoxia. This hypothesis was supported by their observation that HCT-116 cells with knocked out *p53* changed their hypoxia sensitivity and react in the same

way as HT-29 cells.

To obtain comparable results, a butyrate concentration of 2.5 mM was used in all further experiments, even though this may be suboptimal for HCT-116 cells and treatments of 24 hours. Because HCT-116 cells were always treated less than 24 hours, is was assumed that the butyrate concentration was acceptable.

4.2 Adaption of RT-qPCR method

RT-qPCR is a commonly used technique for measuring quantification of gene expression. For the adaption of a RT-qPCR analysis, a variety of decisions have to be made: when (i) isolating RNA from a sample, (ii) reverse transcribing the RNA into cDNA, and (iii) amplifying the cDNA in a PCR. In the following section, the most important decisions and requirements made in this thesis will be discussed. The discussion is meant to help adapting the developed method for others than the tested dietary fiber.

RNA isolation The preparation of a proper RNA sample is the first fundamental step in adapting a RT-qPCR method. Isolated RNA is strongly exposed to RNA degradation by RNases. For avoiding degradation, RNase inhibitors were used in this thesis. Furthermore, genomic DNA contaminations need to be avoided because these could result in false positive signals in the later PCR. According to McPherson and Møller (2006), genomic DNA can never be completely eliminated from RNA preparations and a non-reverse transcriptase (NRT) sample should be included. Such a sample includes all reagents apart from the reverse transcriptase. A fluorescence signal in this sample indicates a genomic DNA contamination. If the difference in the C_T numbers is greater than six between the target sample and the NRT sample, the genomic DNA contamination will not influence the results of the relative gene expression analysis (Qiagen, 2013). In this thesis, genomic DNA contaminations were eliminated by using a DNase treatment. The effectiveness of the DNase treatment was always evaluated by including a NRT sample in the analysis. In this thesis no genomic DNA contaminations above the influencing threshold limit were found in any of the samples.

The purity and concentration of a RNA sample can be determined by UV-Vis spectrophotometer measurements. In this thesis the UV-Vis spectrophotometer NanoDrop ND-1000 was used. By calculating the ratio of absorbance at 260 nm and 280 nm, the purity of RNA (or DNA) can be estimated. A ratio appreciably lower than the reference (see p. 20) indicates protein contaminations. Furthermore, NanoDrop calculates the ratio of absorbance at 260 nm and 230 nm. A ratio appreciably lower than the reference may indicate contaminations of reagents used in the

RNA isolation. However, based on the experience made in this thesis, the use of these ratios to estimate RNA purity in samples with RNA concentrations below $100\,\mathrm{ng\,\mu L^{-1}}$ was not straightforward. Furthermore, the NanoDrop spectrophotometer can only determine the purity of nucleic acids and cannot distinguish between RNA and DNA. Genomic DNA contaminations are therefore not detectable. For this reason, NanoDrop was primary used for measuring the concentration of the RNA samples.

In this thesis, a RNA isolation kit provided by Qiagen (2012) was used. Several other companies offer kits for the same purpose, for example Agilent Technologies (2010) and Bio-Rad Laboratories (2000). In the kit provided by Qiagen (2012), the biological sample is disrupted and cellular components such as protein, DNA, and RNA are free in solution. Several purification steps are performed for obtaining pure RNA. RNA isolation kits are easy to use by following the provided protocol. Furthermore, the reaction system is thoroughly tested and optimized by the provider which enables optimal RNA yields. A disadvantage of the RNA isolation kit from Qiagen (2012) was the incomplete indication of components in chemical solutions such as buffers. This complicated the understanding of the necessity to use these solutions. When using a RNA isolation kit for the first time it is therefore very helpful to understand as much of the reaction system as possible for preventing contaminations by RNase and genomic DNA.

cDNA synthesis The isolated RNA needs to be reverse transcribed into cDNA since only DNA is a suitable template for later PCR amplification. An important decision in cDNA synthesis is the choice of primers. Advantages and disadvantages when choosing sequence specific, oligo(dT), or random hexamer primers are mentioned on page 8. In this thesis random hexamer primers were used since one of the reference genes was of type rRNA and not mRNA. The poly(A) tail is missing in rRNA and the oligo(dT) primer is therefore not suitable. It can be expected that no specific PCR amplification of the rRNA gene would be possible if oligo(dT) primers were used. Resuehr and Spiess (2003) tested the effect of oligo(dT) and random hexamer priming and found that a combination of oligo(dT) and random hexamer was the best suitable approach in cDNA synthesis. Therefore, it may be advisable to follow the approach of Resuehr and Spiess (2003) to get the benefit of both primers in future studies.

PCR Another important decision in the RT-qPCR process was the choice of the appropriate temperature and PCR conditions. According to Roux (2009), the choice of not suitable temperature settings may lead to amplification of nonspecific PCR products or no amplification of the desired product. In this thesis, temperature conditions for primer, probe and thermostable DNA

polymerase were used as recommended by the provider of the PCR kit (TaqMan Life Technologies, 2011). The test of the PCR amplification efficiency proved that the chosen temperature conditions were suitable for the PCR.

Equal PCR efficiencies in all reference and target genes are important to allow a reliable comparison between samples. Since the PCR is of exponential nature, differences in the efficiencies can lead to error-prone estimates of expression ratios. The efficiency test is especially important if manually designed primers are used instead of commercial predesigned primers. In this thesis, predesigned TaqMan Gene Expression Assays were used and a measurement of the PCR efficiency is actually not necessary. Thermo Fisher Scientific validated their assays extensively and guarantee a PCR efficiency of 100% ± 10% in samples free of PCR inhibitors (TaqMan Life Technologies, 2012). However, the PCR efficiencies of the candidate reference genes were determined nonetheless in order to check whether the PCR conditions were optimal. An efficiency between 90% and 110% is supposed to be optimal for PCR (Garson et al., 2009). All tested reference genes achieved PCR efficiencies in this range, except for *18S rRNA* which was slightly out of range with 111%. For estimating the efficiency, standard curves were generated but these standard curves consisted of only three points in a 10-fold dilution series. This is a quite low number of points and it is more commonly referred to standard curves consisting of five or more points (e.g. Dorak, 2006). The explanatory power of standard curves with three points is lower than those with five or more points. Given that the efficiency of *18S rRNA* is just marginally out of range and the test was based on small dilution series, it was nevertheless accepted as sufficient. The PCR efficiency for the three target genes *NF-kB1*, *PTGS2*, and *NFE2L2* was assumed to be 100% ± 10% as guaranteed by Thermo Fisher Scientific and not further analyzed. The results of the PCR efficiency revealed, that the PCR conditions were sufficient.

All the decisions and requirements mentioned above are important for analyzing the stability of reference genes. Unfortunately, these information are often not mentioned in the literature.

4.3 Stability of reference genes

Comparison with other studies Reference genes used for normalization in RT-qPCR need to fulfill several criteria. One criterion is the stable expression under different experimental treatments. The expression stability can be determined by using different statistical and mathematical algorithms. In this thesis, four reference genes were tested for their stability under the experimental conditions by using three different validation methods as advised by Kozera and Rapacz (2013). The four reference genes were *GAPDH*, *18S rRNA*, *B2M*, and *ACTB*. In this section the findings of other studies that evaluated the stability of these reference genes will be discussed.

Because *GAPDH* is one of the most commonly used reference genes in RT-qPCR in general (e.g., Edwards and Denhardt, 1985; Winer et al., 1999) it was also used. In the course of this thesis, it turned out that *GAPDH* is often described as unstable in studies with colorectal cancer tissue and colon cancer cell lines. Dydensborg et al. (2006) showed altered *GAPDH* gene expression between normal and colon tumor tissue. Ersahin et al. (2014) worked with human cancer cell lines and described novel reference genes. They report that *GAPDH* was a stable reference gene in liver cancer cell lines but not in breast and colon cancer cell lines.

The study of Ersahin et al. (2014) showed that *ACTB* was a stable reference gene in colon cancer cell lines. This was confirmed by Duary et al. (2014) who evaluated *ACTB* as one of the most stable reference genes in a study were the expression of pro-inflammatory and anti-inflammatory genes in HT-29 cells were tested on two potential probiotic strains.

The studies of Dydensborg et al. (2006) and Kheirelseid et al. (2010) showed the suitability of *B2M* as reference gene. Dydensborg et al. (2006) found that *B2M* was the best-suited gene for normalization when the objective was to analyze the gene expression between healthy and adenocarcinomas in the human colon. *B2M* was one of the two most stable reference genes out of 13 in the study of Kheirelseid et al. (2010) who evaluated suitable reference genes for comparing primary colorectal tumor and tumor associated normal tissue.

Several studies advised to use the reference gene *18S rRNA* with care. Dijkstra et al. (2014) critically reviewed quantitative PCR results in colorectal cancer research and pointed out that *18S rRNA* should only be used as reference gene after an accurate validation process. Furthermore, it should never be used as single reference gene. One problem of using *18S rRNA* as a reference gene is that the rRNA synthesis is independent of the synthesis of mRNA due to different RNA polymerases. This may result in differences in expression patterns between the RNAs transcribed by different RNA polymerases (Radonić et al., 2004).

Methodological aspects Three different statistical methods were used to analyze the stability of the potential reference genes. In this section, the advantages and disadvantages of the methods will be discussed. No significant differences in gene expression of the four reference genes were found in HT-29 cells after different treatments when analyzing the data with ANOVA and Tukey HSD tests. In HCT-116 cells the ANOVA and Tukey HSD test suggest that *GAPDH* and *18S rRNA* were not expressed in a stable manner under the experimental treatments. One possible explanation for the observed instability of *18S rRNA* and *GAPDH* in HCT-116 but not HT-29 cells can be the lower number of observations in HCT-116 cells (n=4) compared to HT-29 cells (n=6).

The stability of the four reference genes was further tested by using two of the most commonly

used free software programs, BestKeeper and NormFinder. The two approaches did not rank the stability of the reference genes in the same way. According to the BestKeeper software, the most stable reference gene was *B2M* in HT-29 cells and *18S rRNA* in HCT-116 cells. Opposed to that, the NormFinder software identified *18S rRNA* as the most stable reference gene in HT-29 cells and *ACTB* in HCT-116 cells. Both, BestKeeper and NormFinder, identified *GAPDH* as the least stable reference gene in HT-29 and HCT-116 cells. According to Anstaett et al. (2010), the different results in the BestKeeper and NormFinder software occur due to different algorithms used in the calculation of the gene stability. There is no consensus in literature whether BestKeeper or NormFinder is preferable for validating the stability of reference genes. Both are however better suited than the ANOVA and Tukey HSD test because they consider the relation between all reference genes. Since BestKeeper and NormFinder generated similar results for the most stable reference genes in this thesis, it was decided that the arithmetic mean of *18S rRNA*, *B2M*, and *ACTB* was best suited for normalization.

While ANOVA and Tukey HSD tests may be seen as a simple pre-analysis step for validating the reference genes, the main focus should be put on the results of NormFinder and BestKeeper. All statistical tests require enough observations to obtain reliable results. The number of observations in this thesis are rather small and future studies would profit from more observations.

Normalization After validating the reference genes with the statistical approaches discussed above, *GAPDH* was found to be not optimally suited as reference gene. This observation brings up the question of how reference genes that are less stable than they are supposed to be, influence the normalization process. This question is of importance, if a proper validation of reference genes is ignored (e.g., due to budgetary constraints). To test the effect of unstable reference genes in a gene expression experiment, different combinations of reference genes, including the unstable *GAPDH*, were used for normalization in this thesis.

In HT-29 cells, the same results were obtained when the relative expression of the target genes was determined by using the $2^{-\Delta\Delta C_T}$ method, regardless of using the arithmetic mean of the three most stable reference genes (*18S rRNA*, *ACTB*, and *B2M*), the arithmetic mean of all reference genes, or only *18S rRNA*. However, when using only *GAPDH* as a single reference gene in normalization, changes in gene expression in *NFE2L2* due to the different treatments were not detectable. This observation points out the risk of using unstable reference genes for normalization. This problem was not observed for HCT-116 cells for which the stability of the reference genes was more homogenous.

In HT-29 cell experiments with three and six hours treatment time only the stability of

18S rRNA and *GAPDH* was analyzed. The use of *18S rRNA* as single reference gene for normalization was suitable for these experiments as the results were in accordance with the results of using the arithmetic mean of three reference genes.

4.4 Suitability and evaluation of the developed test system

The aim of this thesis was to establish a test system for studying specific molecular mechanisms behind the potential protective effect of certain dietary fibers and their fermentation metabolites on the development of colon cancer. Up- and down-regulations of individual target gene expressions were observed after treatment with dispersible wellmune or butyrate.

One of the target genes was the transcription factor NF-κB, which is one of the key regulators in innate immune response and inflammation (Sakamoto et al., 2009). The activation of the NF-κB transcriptions factor promotes proliferation and prevents apoptosis of cancer cells (Hassanzadeh, 2011). The down-regulation of *NF-κB1* gene expression observed in HT-29 cells after 24 hours incubation with dispersible wellmune or butyrate in this thesis may therefore be one potential mechanism by which dietary fiber protects against the development of colon cancer. As a key regulator of innate immunity, NF-κB is also deeply involved in inflammation. Also in this respect a down-regulation of *NF-κB1* gene expression and in turn NF-κB activity may be protective, since chronic inflammation is associated with the development of cancer (Balkwill and Mantovani, 2001; Coussens and Werb, 2002) and tumors create a pro-inflammatory environment to promote progression and metastasis (Nelson and Ganss, 2006). An inhibition of NF-κB has been suggested as a useful anti-tumor treatment and Sakamoto et al. (2009) found that the tumor expansion was suppressed in *in vivo* cells with inhibited activation of NF-κB. The findings of this thesis are in agreement with Yin et al. (2001) who showed that butyrate inhibits the activation of NF-κB in HT-29 cells. An inhibitory effect of dispersible wellmune on NF-κB activation has previously been shown by Shah (2009) in microglia (primary immune cells of the central nervous system).

Another target gene was *PTGS2*, which is one of the genes regulated by NF-κB. PTGS2 is involved in the development of cancer, cell transformation, and tumor growth (Chandrasekharan and Simmons, 2004). Furthermore, *PTGS2* is over-expressed in colorectal cancer (Eberhart et al., 1994). It was shown that PTGS2 inhibitors inhibit intestinal polyps and colorectal tumors (Steinbach et al., 2000). In this thesis, the gene expression of the target genes *NF-κB1* and *PTGS2* was down-regulated in HT-29 cells after treatment with butyrate or dispersible wellmune for 24 hours. In the light of the studies cited above, these findings indicate that butyrate and dispersible wellmune may act as anti-inflammatory agents in the tested human colon carcinoma cell lines.

The third target gene in this thesis encodes the transcription factor NFE2L2, which regulates

more than 100 genes (Sporn and Liby, 2012). NFE2L2 has a protective effect in suppressing oxidative stress and inhibits carcinogenesis (Ramos-Gomez et al., 2001). There are abundant indications that the activation of NFE2L2 suppresses carcinogenesis in an early state (Hayes et al., 2010). Furthermore, drugs that enhance the activation of NFE2L2 have been shown to prevent cancer (Kwak and Kensler, 2010). Taken the above mentioned studies as a basis, an up-regulation in *NFE2L2* gene expression indicates an anti-oxidative effect. In this thesis, the gene expression of *NFE2L2* was up-regulated in HT-29 cells after 24 hours treatment with dispersible wellmune. No significant difference in gene expression was observed for the butyrate treatment after 24 hours.

All the observed effects of dispersible wellmune and butyrate were dependent on the treatment time. In HT-29 cells, three different treatment times were tested. The results clearly showed that the different effects on target gene expression occurred at different time points. While the inhibitory effect of butyrate on *NF-κB1* and *PTGS2* gene expression was already detectable after three hours, the inhibitory effect of dispersible wellmune on the expression of those two genes needed 24 hours to fully develop. The stimulating effect of dispersible wellmune on *NFE2L2* gene expression, on the other hand, was already detectable after six hours. Incubation of HT-29 cells with butyrate for six hours resulted in inhibition of *NFE2L2* gene expression, an effect that was not observed with three or 24 hours incubation. These results show that the incubation time has to be carefully selected in order to fit the right "time-window" of the response. In this thesis, the effects observed on all target genes by the two treatments at earlier time points were still apparent after 24 hours incubation with the exception of the inhibitory effect of butyrate on *NFE2L2* after six hours. An incubation time of 24 hours seems therefore to be sufficient to detect the majority of changes in gene expression and can be used as a screening incubation time in future studies.

For the HCT-116 cell line, only six hours with butyrate or dispersible wellmune treatment were tested. The results of the six hours treatment time in HCT-116 cells were similar to the results of the six hours treatment time in HT-29 cells. However, there was one exception. The C_T values of *PTGS2* in HCT-116 cells were outside the cut-off level for TaqMan gene expression assays. This observation indicates that *PTGS2* is not or only in very low abundance expressed in this cell line. This is in agreement with the findings by Park et al. (2003) who also found that *PTGS2* is not expressed in HCT-116 cells. Due to different mutations in the HT-29 and HCT-116 cell line, they should represent two different colon cancer cell line models. However, both cell lines revealed the same results in this thesis and it may be considered to use only the HT-29 cell line in future studies because HCT-116 does not express all of the target genes.

As the developed test system was able to detect the potential protective effects of the specific dietary fiber dispersible wellmune and the known fiber metabolite butyrate, it may be well suited

as a screening system for different dietary fibers and their metabolites.

5 Conclusion and future perspectives

The aim of this thesis was to establish a test system to study the protective effect of dietary fibers and their metabolites against colon cancer by using gene expression analysis. Therefore, a RT-qPCR method was adapted for gene expression analysis in HT-29 and HCT-116 cells. For the correct application of this method the experimental setups of all different steps had to be optimized. In conclusion, the following steps were found to be important. A sterile technique during RNA isolation and the use of DNase proved to be important for obtaining RNA templates preparations free from RNase or genomic DNA contaminations. The use of correct dilutions and right choice primers was important for obtaining good quality cDNA. An important consideration for analyzing the RT-qPCR data was the identification of valid reference genes. For this purpose, the application of the freely available validation programs BestKeeper and NormFinder was of benefit. The target genes chosen in this thesis were an initial starting point for establishing the test system. They were chosen because of their roles in inflammation (*NFκB1* and *PTGS2*) or inhibition of carcinogenesis (*NFE2L2*). The down-regulation of *NFκB1* and *PTGS2* and up-regulation of *NFE2L2* gene expression observed in HT-29 cells treated with dispersible wellmune or butyrate may be one potential mechanism of dietary fiber's protective effect against the development of colon cancer.

Based on these results it seems likely that the established test system will be able to give new insights into the molecular mechanisms of this protective effect and at the same time enable the selection of dietary fiber types or metabolites with the highest protective potential in future studies. The development of colon cancer is associated with a change in the expression of plenty of genes. It would be interesting to further study dietary fiber types using the established test system and to apply a DNA microarray analysis to the fiber types with the strongest effects. In this way the expression levels of several thousand genes treated with the specific dietary fiber can be studied at the same time. If further dietary-fiber regulated genes would be found, patterns and relations between the genes could be analyzed. These patterns may help to understand more of the molecular mechanisms involved in the protective effects of the respective dietary fiber types.

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